ORGANISATION OF DIGESTION AND DIGESTIVE PROTEINASES IN THE GREEN MIRID, *CREONTIADES DILUTUS.*

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A thesis submitted for the degree of Doctor of Philosophy

at the Australian National University.

This thesis does not contain any material that has been accepted for the award of any other degree or diploma at this or any other University. To the best of my knowledge, this thesis does not contain any material that has been published previously, except where due reference is made in the text. The research described in this thesis is my own original work with the following exceptions:

Dr. Paul Cooper and Neda Plovanic embedded, sectioned and photographed midguts for microscopy, and the midguts and salivary glands for transmission electron microscopy (Chapter 2). The degenerate primers for the amplification of serine proteinase genes were designed by Dr. Peter East and Irandokt Zolfaghar (Chapter 4).

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ABSTRACT

This thesis has examined digestion in a predominantly phytophagous hemipteran insect, the green mirid, *Creontiades dilutus* (Heteroptera: Cimicomorpha: Miridae). The structure of the green mirid digestive system was examined and found to be typical of insects within the infraorder Cimicomorpha. The salivary glands are large and are made up of an accessory gland and a principal gland on each side of the gut. The principal gland is divided into two lobes which differ in structure and function, with the posterior lobe being the site of the majority of proteinase activity. The gut of the green mirid is dominated by the mildly acidic midgut, which is comprised of an anterior sac-like region and two posterior tubular sections divided by a constriction. The cells of the midgut are all of one structural type and are discontinuously lined by extensive extracellular membrane layers. There is ultrastructural evidence that the anterior region of the midgut is more active in storage and secretion than the posterior region.

Amylase and proteinase activities were measured in the digestive tissues and secreted saliva of the green mirid using general substrate assays, and both were found to be more active in the salivary glands than in the midgut. Proteinase activity as detected by azocasein was quite low and did not differ between male and female mirids, adults and third instar nymphs, or fed and starved insects. In the salivary glands and secreted saliva, serine proteinases, specifically chymotrypsin-like, predominated, based on inhibitor specificity, basic pH optima (pH 8), and hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanalide and *N*-succinly-ala-ala-pro-leu *p*-nitroanalide. The pH optimum of midgut extracts was acidic (pH 4), suggesting acidic proteinases predominate. However, proteinase activity was substantially inhibited by both aprotinin and E-64, implying the presence of both serine and cysteine proteinases. The presence of multiple bands of activity on zymograms also suggested that several proteinases are active in the digestive system of the green mirid.

Molecular analyses were consistent with the biochemistry results. RT-PCR with degenerate primers succeeded in amplifying three serine proteinases and no cysteine proteinases from salivary gland mRNA, supporting the predominance of serine proteinases in this tissue. One of the serine proteinase amplicons was used to isolate a full length cDNA clone, *CdSp1,* which corresponds to a putative chymotrypsin-like gene. *CdSp1* is expressed at high levels in the posterior lobe of the principal salivary gland, and not at all in the anterior lobe of the principal salivary gland, the accessory gland, or the midgut. RT-PCR was also used to identify two serine serine proteinase genes, one of which encodes a non-functional protein, and three cysteine proteinase genes, from the midgut of the green mirid, supporting the assertion that the midgut of the green mirid contains a complex mixture of digestive proteinases.

The possible effect of the high levels of enzyme activity in the salivary glands relative to the midgut on pre-oral digestion are discussed. The implications of the range of proteinases observed in the digestive system of the green mirid are also examined, with particular focus on the evolution of heteropteran insects

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LIST OF ABBREVIATIONS

AEBSF; 4-(2-Aminoethyl)-benzenesulfonylfluoride, hydrochloride (Pefabloc® SC)
ANOVA; analysis of variance
antipain; antipain-dihydrochloride
BApNA; *Nα*–benzoyl-DL-arginine *p*-nitroanilide
BCIP; 5-Bromo-4-chloro-3-Indolyl Phosphate
BTpNA; *N*-benzoyl-L-tyrosine *p*-nitroanalide
DIG; digoxygenin
DMF; dimethylformamide
DTT; dithiothreitol
ECML; extracellular membrane layers
EDTA; ethylenediamine tetraacetic acid
EGTA; ethylene glycol-bis(β-aminoethyl ether)
EM; electron microscopy
h; hour
HRP; horse radish peroxidase
min; minute
MW; molecular weights
NBT; Nitro blue Tetrazolium
PBS; phosphate buffered saline
PCR; polymerase chain reaction
pNA; *p*-nitroanalide
RT-PCR; reverse transcription polymerase chain reaction
SA_2PLNA; *N*-succinyl-ala-ala-pro-leu *p*-nitroanilide
SA_3pNA; *N*-succinyl-ala-ala-ala *p*-nitroanilide
SDS; sodium dodecyl sulfate
SE; standard error
TCA; trichloroacetic acid

LIST OF SOLUTIONS

Insect saline solution; 0.1 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $NaHCO_3$, pH 7
Phadebas assay buffer; 100 mM Na-succinate, 20 mM $CaCl_2$, pH 6.5
Sample buffer (2 x); 0.0625 M Tris.HCl, 10% Glycerol, 0.0125% Bromo-phenol Blue, 4% sodium dodecyl sulfate
Running buffer; 1.5% Tris, 7.2% Glycine, 0.5% SDS (w/v)
Refolding buffer; 0.05 M Tris, 0.2 M NaCl, 0.55% $CaCl_2$, 0.067% (w/v) Brij35, pH 7.6

CHAPTER 1: INTRODUCTION

1.1 EVOLUTION OF THE HEMIPTERA

The order Hemiptera is an extremely successful order. There are more than 5,650 species of Hemiptera in Australia alone, and they can be found in almost all ecological niches (Carver *et al.*, 1970). There are four suborders currently recognised within the Hemiptera; the Sternorrhyncha, Cicadomorpha, Fulgoromorpha and Heteroptodea (Sorenson *et al.*, 1995) (Fig. 1.1). The mouthpart structure is uniform throughout the four suborders, and is well adapted to a fluid diet (see section 1.2.1). This uniformity implies that the mouthpart structure was fully evolved before radiation into the suborders, and that the ancestral hemipteran was a fluid feeder (Goodchild, 1966). The ability of the Hemiptera to feed on a fluid diet has allowed the exploitation of many different food sources. The first three suborders (Sternorrhyncha, Cicadomorpha and Fulgoromorpha) are all sap-suckers, which feed from the vascular tissues of plants. The suborder Heteroptodea is by far the most diverse group. It comprises the plant cell feeding infraorder Coleorrhyncha, and the Heteroptera clade, which includes infraorders containing semi-aquatic predators, terrestrial predators, blood-suckers, seed feeding insects, plant cell feeders and true sap-suckers (Goodchild, 1966).

Hemiptera use one of five different modes of feeding (Miles, 1972; Cobben, 1978). The first is the primitive scratch-and-suck method, probably used by the modern Tingidae (Heteroptera), which involves scratching at the surface cells of the plant and sucking up the contents. The second is stylet-sheath feeding, so termed because during feeding a salivary secretion is produced which then solidifies around the mouthparts to form a tubular sheath. Stylet-sheath feeding is used by all three sap-sucking suborders, as well as the sap-sucking insects of the infraorder Pentatomomorpha (Heteroptera). Some predatory Heteroptera also use this mode of feeding, except the secretion is limited to the outside of the prey, forming a salivary flange rather than a tubular sheath (Cohen, 1990). The third mode of feeding, lacerate-and-flush, is used by both seed and plant cell feeding Heteroptera and involves the

secretion of a watery saliva into the food to externally digest pockets of cells. The fourth mode of feeding is predation, which is essentially the same as lacerate-and-flush feeding except that the composition of the secreted saliva is different, and the food source is animal rather than plant tissue. The final mode of feeding is blood-sucking, where a blood vessel of the vertebrate host is penetrated by the stylets, but little secretion of salivary hydrolases occurs.

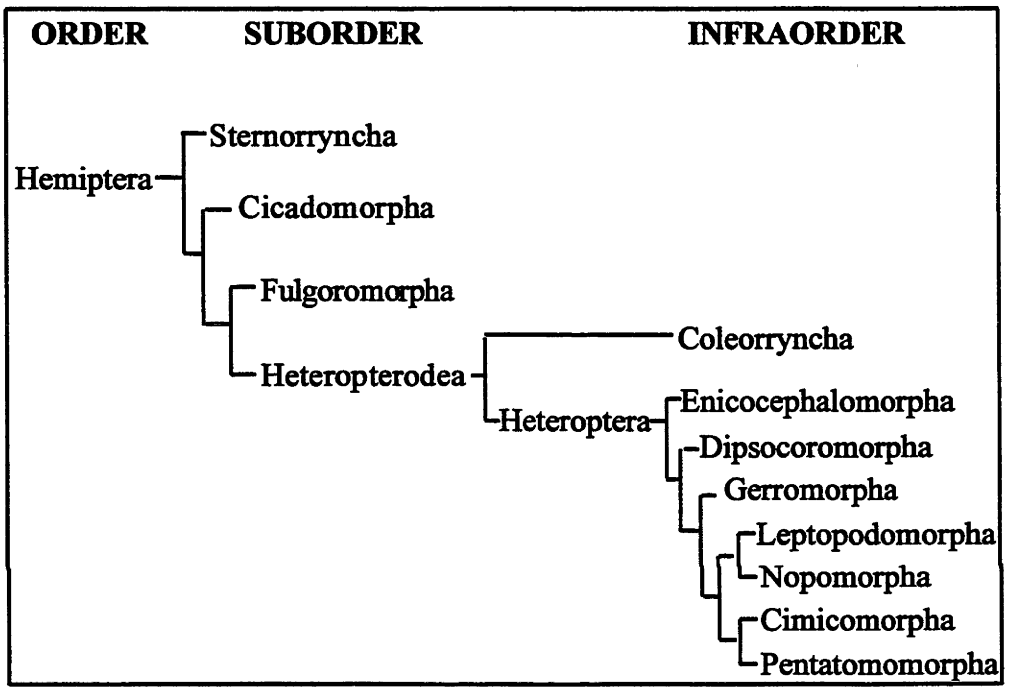


Figure 1.1: Phylogeny of the Hemiptera (after Sorenson *et al.*, 1995)

There has been considerable debate over the possible feeding style and food source of the ancestral Hemiptera. It has been suggested that the pre-Hemiptera could have been predators (Cobben, 1978), although it is more widely accepted that they were phytophagous. Yet there is still some confusion in the literature as to whether the plant material ingested was mesophyll cells (Sweet, 1964; Goodchild, 1966) or phloem material (Houseman and Downe, 1983; Terra, 1990). However, the great variation in gut structure among sap-sucking Hemiptera implies sap-sucking arose independently more than once, and was not a common feature of the ancestor (Goodchild, 1966).

Some debate also surrounds the possible feeding habits of the ancestral Heteroptera. It has been proposed that the progenitor may have been fungivorous (Elson, 1937), omnivorous (Goodchild, 1966), carnivorous (Cobben, 1978) or phytophagous (Sweet, 1979). It is most likely that the Heteroptera were either plant-feeding or carnivorous from origin, but it remains unresolved as to which. Evidence for carnivory in the ancestral Heteroptera includes feeding apparatus and current food sources. While the general mouthpart structure is conserved from the hemipteran ancestor, specific adaptations to predation, such as heavily barbed maxillae, are present in almost all heteropterans and so presumably arose before divergence into the different species and feeding types (Cobben, 1978). In addition, carnivory is very common in the Heteroptera, with approximately half of the heteropteran families being exclusively carnivorous. This evidence suggests that the ancestral heteropteran was at least partially predaceous (Cobben, 1978; Goodchild, 1966).

To explain this change in lifestyle from phytophagy to one which included predation, Goodchild (1966) proposed that the ancestral Heteroptera moved from plants to become more active within the forest floor litter (Fig. 1.2). The plant diet was thus abandoned for a more generalised diet, including much more animal life. Presumably this change in habitat and diet resulted in the adoption, at least in part, of a predatory lifestyle. The assumption of the original leaf litter habitat is based on the fact that the structure of heteropteran mouthparts is suited to leaf litter dwelling, and on the fact that the most primitive Heteroptera continue to live in this environment (Goodchild, 1966).

Most modern Infraorders of the Heteroptera are semi-aquatic or aquatic and exclusively carnivorous, with the notable exceptions of the mostly terrestrial Cimicomorpha and Pentatomomorpha. The Cimicomorpha are predominantly carnivorous, but include some taxa of phytophagous insects. Conversely, the Pentatomomorpha are predominantly phytophagous, including seed feeders and some true sap-suckers, but also include a few groups of carnivorous insects. Marked differences in the structures of both the salivary glands (Miles, 1972) and the intestines (Goodchild, 1963) of the Cimicomorpha and Pentatomomorpha have been observed. This suggests that these two groups split early in the history of the Heteroptera, with

the Cimicomorpha remaining carnivorous and the Pentatomomorpha reverting to phytophagy (Goodchild, 1963). If so, this implies that both the phytophagous Cimicomorpha and the carnivorous Pentatomomorpha have arisen relatively recently. Certainly there is evidence that predation is more recently evolved in the Pentatomomorpha than in the Cimicomorpha (Cohen, 1998).

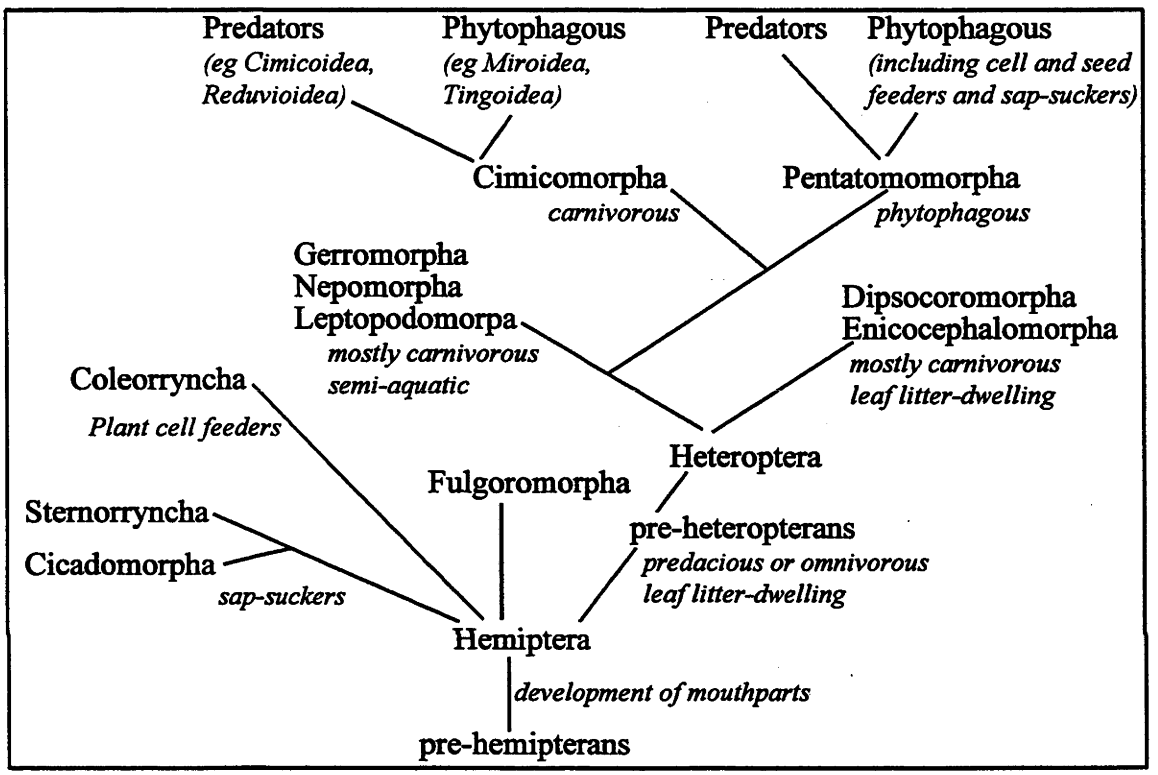


Figure 1.2: Proposed evolution of the Hemiptera (after Goodchild, 1966)

1.2 STRUCTURE AND FUNCTION OF THE DIGESTIVE SYSTEM OF THE HEMIPTERA

1.2.1 Mouthparts and Foregut

As previously noted, the mouthparts of all hemipterans are extremely conserved and well adapted to a fluid feeding diet. The mandibles and maxillae are modified to form two pairs of flexible stylets, which lie in a groove in the labium, and are partially protected anteriorly by the labrum (Fig. 1.3 A). The mandibular pair of stylets

surround the maxillary pair, and longitudinal grooves in the maxillary stylets form the food canal and the salivary canal (Cobben, 1978) (Fig. 1.3 B). The needle-like stylets are used to penetrate the food source. In most Heteroptera, watery saliva containing a mixture of digestive enzymes is secreted via the salivary canal to aid penetration of the food and to begin digestion externally (Miles, 1972; Cohen, 1990) (see section 1.2.4). Partially liquefied food is sucked in through the food canal, which, along with the salivary canal, opens posteriorly into the pre-oral cavity or pharynx. This region is associated with powerful dorsal muscles to control the pumping out of saliva and the subsequent sucking in of food (Elson, 1937). The pharynx joins posteriorly to the oesophagus. The oesophagus in most hemipterans is narrow and thin walled, and opens directly into the midgut through the oesophageal valve (Goodchild, 1966).

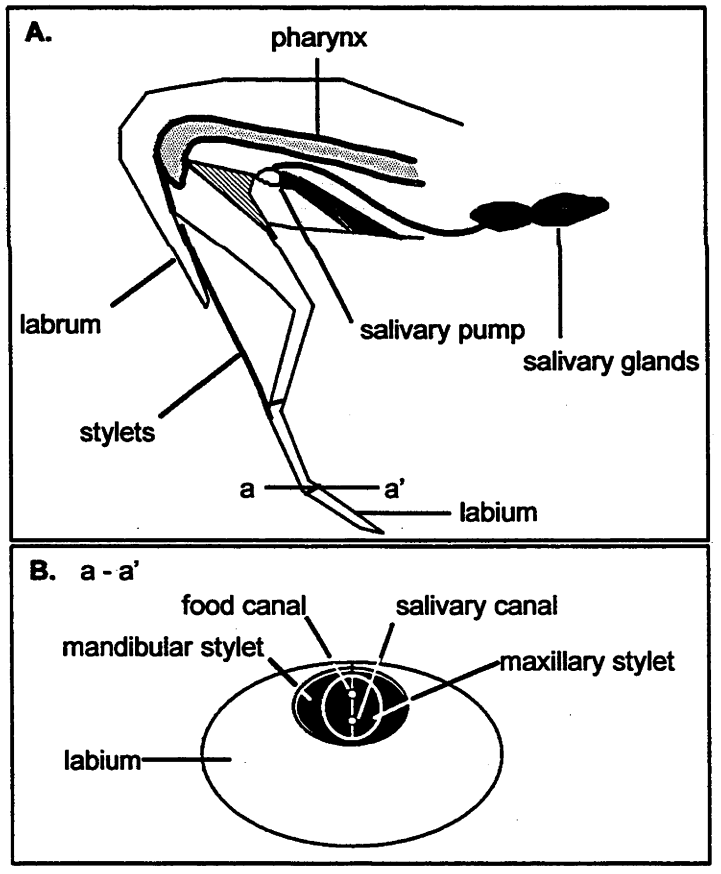


Figure 1.3: A. Mouthparts of the Hemiptera, and **B.** Transverse section through hemipteran stylets (section a-a' in Fig. 1.3 A) (Carver *et al.*, 1970)

1.2.2 Gut

1.2.2.1 General Hemipteran Gut

There is apparently no crop in hemipteran insects, so that the oesophagus constitutes the entire foregut (Goodchild, 1966). The oesophagus leads directly into the slightly acidic midgut, which forms the majority of the intestine, and where storage, digestion and absorption of food all occur. The midgut of the Hemiptera has unique features which appear to be adaptations to the simplicity of a fluid diet, and which mostly result in a reduction in structural complexity. Both anterior gastric caecae, which can increase the surface area of the midgut, and a peritrophic membrane, are apparently lacking in hemipteran guts (for example see Kershaw, 1931; Cheung and Marshall, 1973; Ponsen, 1992; Goodchild, 1963). The peritrophic membrane is a chitinous, perforated membrane, which encloses the food in the lumen of the guts of most insect Orders, and which is thought to primarily function in protecting the midgut cells by acting as a barrier against the passage of micro-organisms, digestive enzymes, and the products of digestion (Terra, 1990). The gap between the peritrophic membrane and the cells of the gut, the ectoperitrophic space, is involved in the compartmentalisation of digestion and possibly in establishing a countercurrent of fluid in insects (Terra, 1990).

While apparently lacking a true peritrophic membrane, the midgut cells of most hemipteran insects are covered by a system of membranes which are proposed to have been aquired in the ancestral hemipteran to increase the absorption of nutrients from a very dilute diet (Ferreira *et al.*, 1988). These membranes ensheath the microvilli forming an outer (perimicrovillar) membrane which maintains a constant distance from the inner microvillar membrane (Lane and Harrison, 1979; Billingsley and Downe, 1988; Silva *et al.*, 1995). The perimicrovillar membranes extend into the lumen to form a system of extracellular membrane layers, which have been most extensively studied in *Rhodnius prolixus* where they are produced particularly in the posterior midgut over a period of 20 days, beginning 2 days after a blood meal (Billingsley and Downe, 1983). The perimicrovillar membranes are involved in the absroption of nutrients including amino acids through the use of active transport to establish a

concentration gradient (Terra, 1990). In addition, they have been described as a modified peritrophic membrane (Lane and Harrison, 1979), and enclose a space, termed the perimicrovillar space, which may compartmentalise digestion in a similar way to the ectoperitrophic space (Terra, 1990), thus concentrating digestive enzymes near the microvilli (Billingsley and Downe, 1983) and maximising the absorption of digested nutrients (Ferreira *et al.*, 1988).

There are four Malpighian tubules present in most hemipteran digestive systems (Goodchild, 1966). These tubules are usually thin walled with a narrow lumen that opens into the extreme posterior midgut, or pylorus. The pylorus is not differentiated from the rest of the midgut in the Sternorrhyncha, Cicadomorpha, or Fulgoromorpha, but in the Heteroptera it is separated from the gut anteriorly by a valve-like constriction and posteriorly by the pyloric valve, which marks the start of the hindgut (Goodchild, 1963). The rectal gland cells, involved in osmoregulation, can vary in position from lining the entire rectum in some groups, to being limited to the anterior hindgut around the pyloric valve in other groups.

1.2.2.2 The Sap-Sucking Hemiptera

Within families, and to some extent within infraorders, some uniformity of gut structure is usually seen. However, gut structure varies enormously among the higher taxa of the Hemiptera. The most unique features of the guts of the sap-sucking orders are the adaptations which allow maximal extraction of nutrients from, and prevent dilution of the haemolymph by, the very dilute food source. The presence of a filter chamber is the most common adaptation to a sap diet. Two main types of filter chambers have arisen, the basic concept of both being the passage of water directly from the anterior to the posterior of the gut, without passing through the entire length of the midgut (Goodchild, 1966). In this way the solutes within the midgut are concentrated.

In most Sternorrhyncha, the anterior and posterior regions of the midgut lie together, sometimes in a very convoluted way, allowing the direct passage of water across the midgut wall. In some Sternorryhncha, the filter system is even more

specialised with the region of contact of the midgut being sunk into the anterior of the rectum (Misra, 1931; Ponsen, 1990) (Fig. 1.4 A). This is probably to separate it from the haemolymph, preventing any leakage of water into the haemocoel. The midgut of the Aphidoidea (Sternorrhyncha) lacks both a filter chamber and Malpighian tubules (Auclair, 1963). The absence of Malpighian tubules allows the build up of pressure in the insect without increasing the loss of solutes, thus enabling a rapid passage of sap through the insect (Goodchild, 1966).

The Cicadoidea (Cicadomorpha) have a classic filter chamber (Fig. 1.4 B). Again the anterior and posterior midgut are in close proximity, this time with the posterior midgut and the Malpighian tubules tracing a complex path within the subperitoneal cavity of the anterior midgut (Cheung and Marshall, 1973). This forms the sac-like filter chamber on the side of the midgut. The Malpighian tubules of the Cicadomorpha often have a wide, glandular segment outside this filter chamber (Cecil, 1930). Water travels passively from the midgut into the Malpighian tubules lying alongside, and then directly to the rectum for excretion (Goodchild, 1966). Upon its emergence from the filter chamber, the midgut, now containing a more concentrated food solution, joins the thin hindgut which extends to the anus.

The gut of the Fulgoromorpha is quite simple and lacks an obvious filter chamber. The midgut is a narrow tube which forms a cluster of loops enclosed by a thin membrane (Kershaw, 1931) (Fig. 1.4 C). This membrane probably functions to prevent the passage of water directly from the midgut into the haemolymph. Another unusual feature of the midgut of the Fulgoromorpha is the presence of an extension, the anterior diverticulum, projecting forward into the thorax, that may be involved in moulting (Goodchild, 1966; Tsai and Perrier, 1993).

1.2.2.3 The Heteroptera

Heteropteran insects are often divided into two main groups; the predominantly phytophagous Pentatomomorpha, and the predominantly zoophagous infraorders, including the Cimicomorpha. The gut of the Pentatomomorpha is usually complex, with the midgut comprising at least three sections, usually including a distinct and large pylorus which may enable more complete reabsorption of solutes from the urine (Miyamoto, 1961; Ahmad *et al.*, 1988) (Fig. 1.5 A). In the sap-sucking families of Pentatomomorpha the gut is even more complex, with an additional section of the posterior midgut displaying several gastric caecae, which often contain bacteria and may be involved in water excretion (Miyamoto, 1961; Al-Sandouk, 1988). The midgut of the Cimicomorpha, however, is a simple tubular organ (Fig. 1.5 B). It comprises only an anterior sac-like region and a posterior tube-like region. In the Bryocorinae (Heteroptera: Miridae) the posterior midgut is further divided into two sections by a constriction (Goodchild, 1963). The pylorus of the Cimicomorpha, unlike the Pentatomomorpha, is extremely small, though still distinct from the rest of the gut. The rectal gland cells of the hindgut are found around the pyloric valve in most Cimicomorpha (Goodchild, 1963).

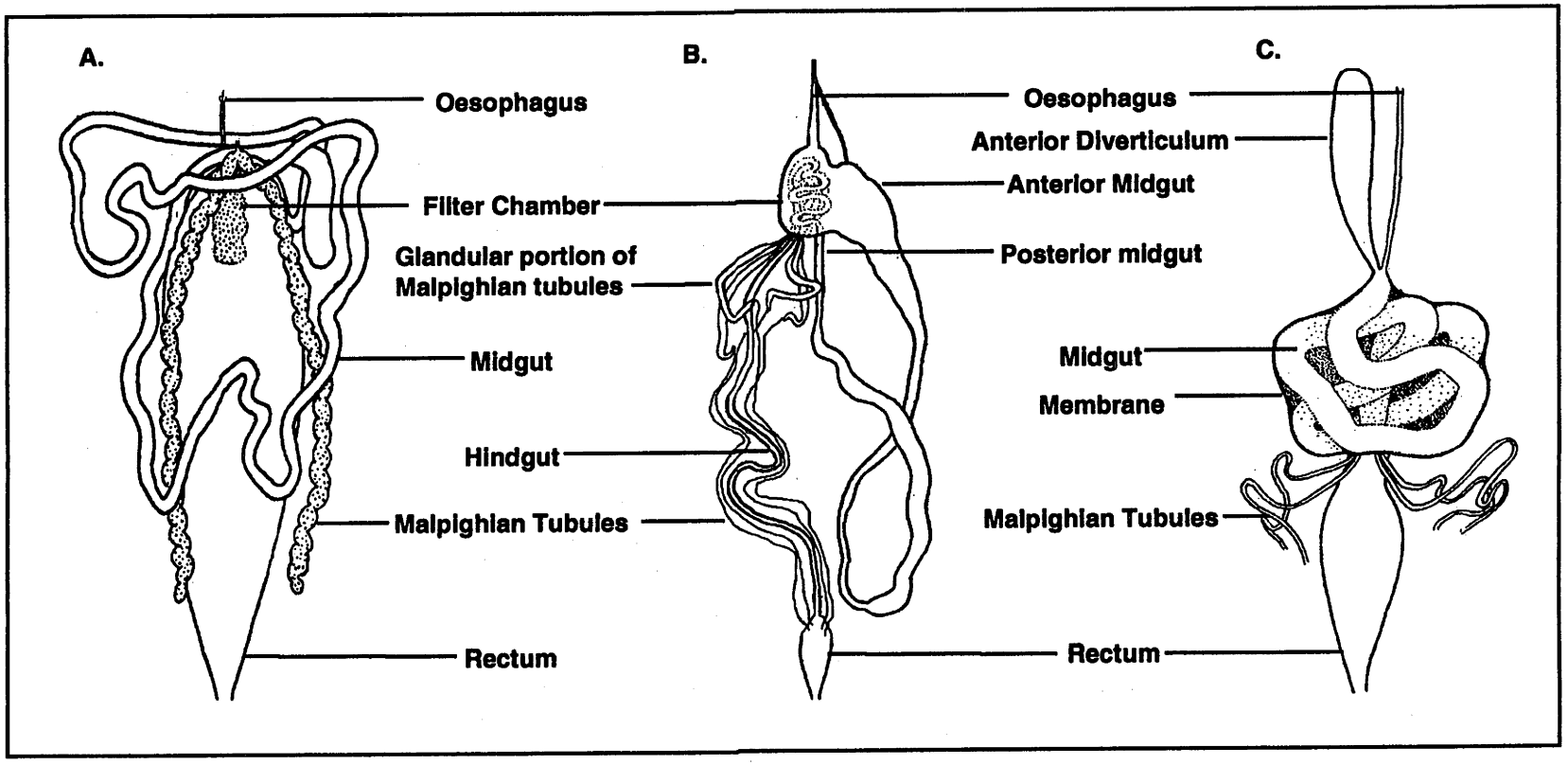


Figure 1.4: Comparison of hemipteran alimentary canals. **A)** *Pulvinaria jacksoni,* (Sternorrhyncha: Coccidae) **B)** *Tettigoniella mitrata,* (Cicadomorpha: Jassidae) **C)** *Pyrops tenebrosus,* (Fulgoromorpha: Fulgoridae) (Goodchild, 1966)

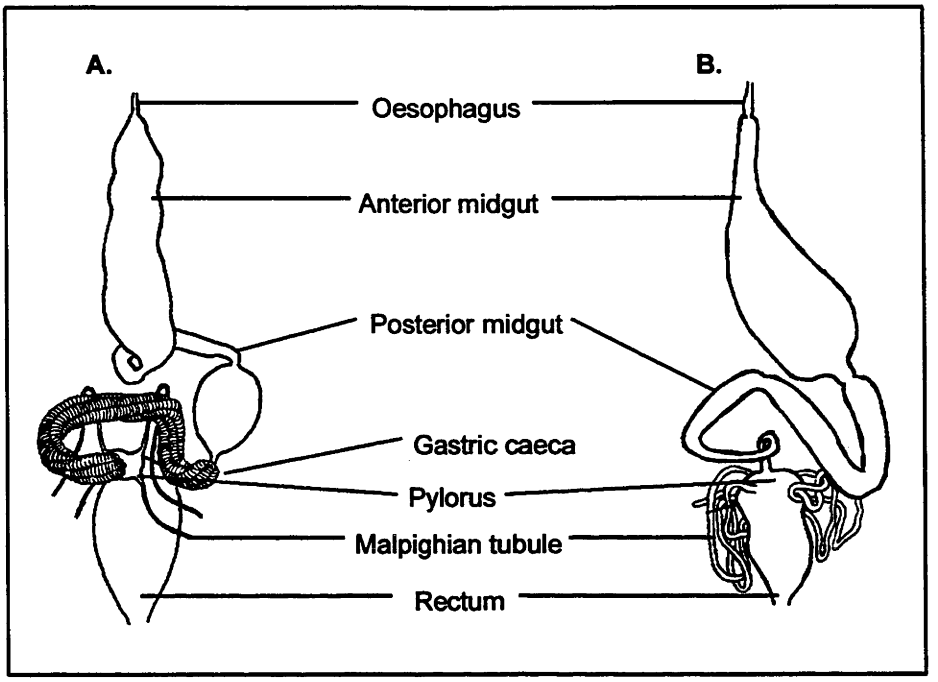


Figure 1.5: Comparison of heteropteran alimentary canals. **A.** *Agonoscelis versicolor* (Heteroptera: Pentatomomorpha) and **B.** *Sahlbergella singularis* Hagl. (Heteroptera: Cimicomorpha) (Goodchild, 1966)

1.2.3 Salivary Glands

1.2.3.1 General Hemipteran Salivary Glands

The salivary glands of the Hemiptera are typically paired and lie in the thorax. Each side comprises a principal gland and duct, and an accessory gland and duct (Miles, 1972) (see Fig. 1.7). The accessory duct joins the principal duct near the principal gland, and the two principal ducts then unite to form the salivary duct which empties into the salivary pump (Miles, 1972). The accessory gland is usually a thin walled bladder, which produces a dilute, alkaline secretion for flushing out food and removing excess water (Miles, 1967). The principal gland is usually larger and more complex than the accessory gland, and is the source of most salivary enzymes (Miles, 1967). Like the midgut, salivary gland structure varies considerably within the Hemiptera.

1.2.3.2 The Sap-Sucking Hemiptera

The principal glands of the Sternorrhyncha, Cicadomorpha, and Fulgoromorpha are, in general, compact groups of cells which enclose a system of channels and ducts comprised of smaller cells (Balasubramanian and Davies, 1968). The overall structure however varies substantially between these groups. The Aphidoidea (Sternorrhyncha) have small but cellularly complex salivary glands, with at least nine different cell types in the principal gland (Weidmann, 1968). The principal gland is partially divided into two halves, and through each half runs a central canal which is continuous with the principal duct (Miles, 1972) (Fig. 1.6 A). The accessory gland is usually small, and functions as the main excretory organ since aphids lack Malpighian tubules (Auclair, 1963). In the Cicadomorpha the principal gland contains at least four and up to 21 cell types, which vary from being grouped together as lobes (*Empoasca fabae* (Cicadomorpha: Jassidae), Berlin and Hibbs, 1963) (Fig. 1.6 B) to remaining separate in the form of rosettes (*Graminella nigrifrons* and *Dalbulus maidis* (Cicadomorpha: Cicadellidae), Tsai and Perrier, 1996). The salivary glands of the Delphacidae (Fulgoromorpha) are quite similar to those of the Cicadomorpha, but usually comprise a larger number of multicellular acini (Miles, 1972) (Fig. 1.6 C). The number of acini is variable, and can vary even within a species, possible due to the age and physiology of the insect (Tsai and Perrier, 1993). Each acini has its own ductule, and the principal duct is formed by the junction of these ductules (Ammar, 1986). An accessory gland is not always distinguishable in the Fulgoromorpha (Miles, 1972).

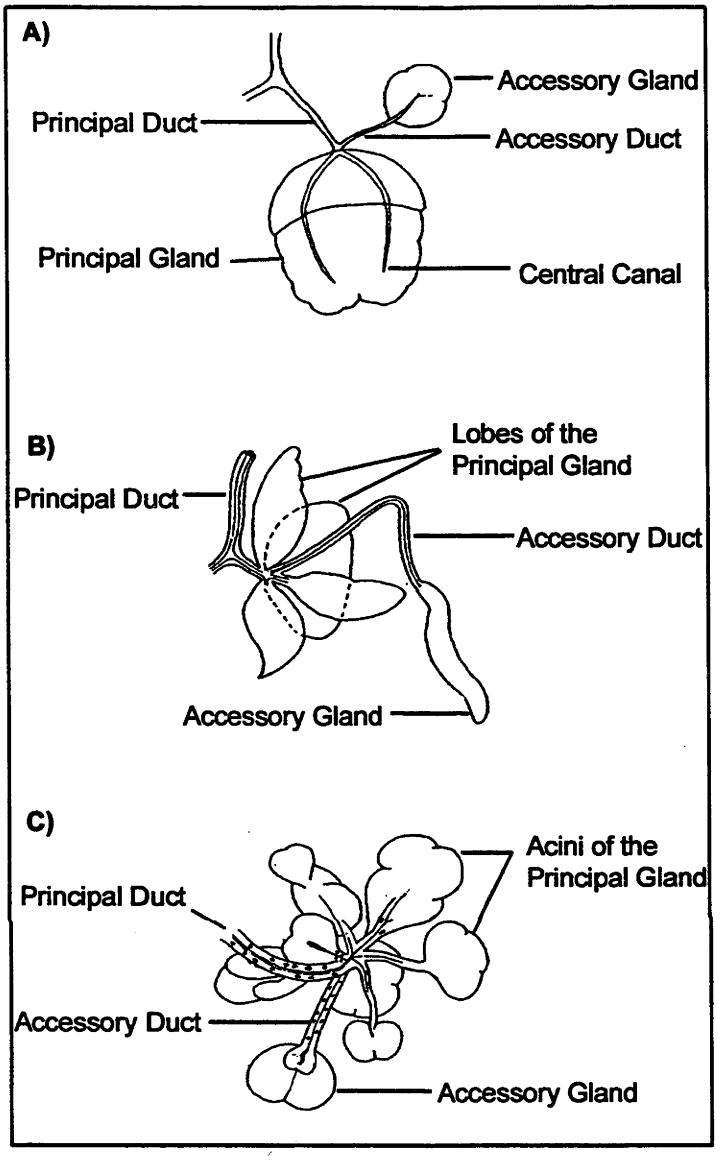


Figure 1.6: Hemipteran salivary glands. **A)** *Myzus persicae* (Sternorrhyncha: Aphidoidea), **B)** *Empoasca fabae* (Cicadomorpha: Jassoidea), and **C)** *Laodelphax striatellus* (Fulgoromorpha: Fulgoroidea) (Miles, 1972)

1.2.3.3 The Heteroptera

The salivary glands of the Heteroptera are generally vesicular, with secretory cells arranged in a layer around a secretion filled lumen, although salivary gland structures within the Heteroptera show great variation. Nuorteva (1956) compared the anatomy of the salivary glands of four families of Heteroptera (Miridae, Lygaeidae,

Coreidae and Pentatomidae), and found that not only did members of the different families vary in gland structure, but that there were notable differences between the subfamilies within the Miridae. In one subfamily, the Bryocorinae, also described in detail by Goodchild (1952), the principal gland consists of two anterior and two posterior lobes, and the accessory gland is heart shaped. In another subfamily, the Mirinae, however, the accessory gland is dumbbell shaped, and the principal gland is bilobed (Fig. 1.7). This is apparently the more primitive form, and the more common form within the Cimicomorpha (Bronskill *et al.*, 1958). In the Pentatomomorpha most families have multilobed principal glands (Miyamoto, 1961), with only a few families such as the Pentatomidae retaining the bilobed form (Baptist, 1941).

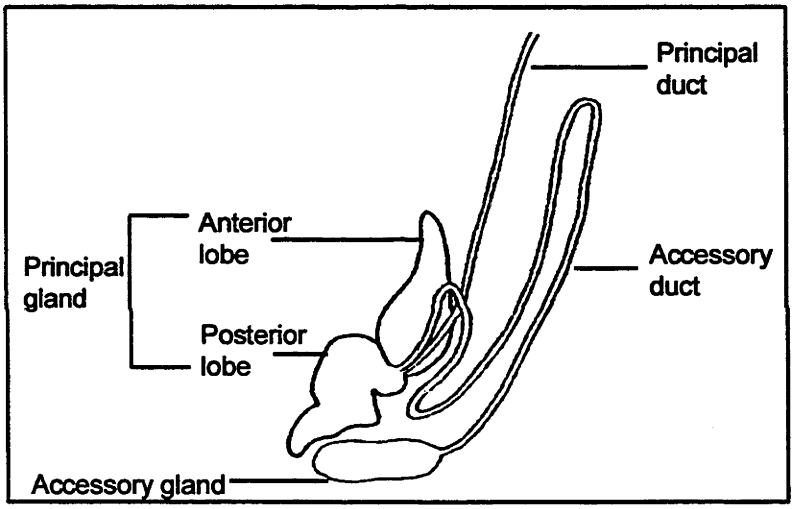


Figure 1.7: Salivary gland of *Lygus pratensis* L. (Heteroptera: Miridae) (Miles, 1972)

Some authors have suggested that the different lobes of the principal gland of hemipteran insects do not perform different functions (Baptist, 1941; Day and Waterhouse, 1953). However, other studies have found that the different lobes do in fact produce different substances and therefore perform different functions (Bronskill *et al.*, 1958; Miles, 1967). The anterior lobe of the pentatomid principal salivary gland was shown to be the source of the sheath material (Miles, 1967). It has recently been demonstrated that some Cimicomorpha families also produce salivary sheath material

(Cohen, 1990). Most Cimicomorpha families however, including Miridae, do not produce a salivary sheath or flange. The anterior lobe in these insects is proposed to function primarily in the production of mucoid substances and lipids to lubricate the stylets and to dislodge particles (Miles, 1972). The posterior lobe of the principal gland in both the Pentatomomorpha and the Cimicomorpha has been reported to be the main source of digestive enzymes (Miles, 1967).

1.2.4 Digestive Enzymes of the Hemiptera

Hemipteran digestive physiology varies among insects of different feeding styles and food sources. For example, hematophages such as *Rhodnius* spp. (Reduviidae) have little proteolytic activity in their salivary glands or anterior midgut, limiting digestion to the posterior midgut (Terra *et al.*, 1988). The seed-feeder *Dysdercus peruvianus* also lacks detectable salivary proteinase activity (Silva and Terra, 1994), while predators such as *Zelus renardii* (Reduviidae) have high levels of proteolytic activity in both the salivary glands and entire midgut (Cohen, 1993). In general, digestion in predatory and phytophagous Heteroptera begins externally through the action of secreted salivary enzymes, and is then completed in the midgut by enzymes produced by the gut cells, and possibly also by ingested salivary enzymes. Most studies of hemipteran salivary enzymes have focussed on members of the families Miridae, particulary the *Lygus* species, and Reduviidae. Many different salivary enzymes have been shown to be active (Table 1.1). Laurema *et al.* (1985) extensively studied the salivary glands of the mirid *Lygus disponsi*, and concluded that the most important salivary enzymes in this insect are pectinase, phospholipase, amylase, and an alkaline proteinase. Pectinase and phospholipase aid penetration of the plant tissue (Strong and Kruitwagen, 1968; Cohen, 1990). While the amylase and alkaline proteinase may also have a role in this, their primary function is probably pre-oral digestion of the plant material (Tingey and Pillemer, 1977). Major components of the midgut digestive enzymes, identified largely from predatory and blood feeding Heteroptera, include proteinase, amylase, and lipase (Table 1.2). It has been suggested that all insects may have the full complement of digestive enzymes, but that different

ones are expressed at different times depending on life stage and diet composition (Terra and Ferreira, 1994), which may explain the wide range of enzymes detected in hemipteran insects.

Table 1.1: Salivary gland enzymes of the Heteroptera

Enzyme	**Family**	**References**
acidic proteinase	Miridae	Kumar, 1970; Varis *et al.*, 1983; Laurema *et al.*, 1985
alkaline proteinase	Belastomadidae	Rees and Offord, 1969
	Lygaeidae	Bronskill *et al.*, 1958; Cohen, 1990
	Miridae	Hori, 1970; Takanona and Hori, 1974; Laurema *et al.*, 1985
	Nabidae	Cohen, 1990
	Pentatomidae	Cohen, 1990
	Reduviidae	Cohen, 1990; Cohen, 1993
amylase	Lygaeidae	Bronskill *et al.*, 1958
	Miridae	Goodchild, 1952; Hori, 1970; Varis *et al.*, 1983; Laurema *et al.*, 1985; Agblor *et al.*, 1994
	Nabidae	Cohen, 1990
	Pentatomidae	Cohen, 1990
glucosidase	Miridae	Laurema *et al.*, 1985
invertase	Miridae	Laurema *et al.*, 1985
pectinase	Miridae	Laurema and Nuorteva, 1961; Hori, 1970; Laurema *et al.*, 1985; Agblor *et al.*, 1994; Cohen and Wheeler, 1998
phosphatase	Miridae	Laurema *et al.*, 1985
phospholipase	Miridae	Goodchild, 1952
	Reduviidae	Cohen, 1990
trehalase	Miridae	Laurema *et al.*, 1985

Table 1.2: Midgut enzymes of the Heteroptera

Enzyme	Family	References
acidic proteinase	Belostomatidae, Lygaeidae, Notonectidae	Houseman and Downe, 1983
	Cimicidae	Houseman and Downe, 1982b
	Pentatomidae	Houseman and Downe, 1983; Houseman *et al.*, 1984
	Phymatidae	Houseman and Downe, 1983; Houseman *et al.*, 1985
	Pyrrhocoridae	Silva and Terra, 1994
	Reduviidae	Houseman, 1978; Houseman and Downe, 1982a; Houseman and Downe, 1983; Terra, 1988; Cohen, 1993
alkaline proteinase	Miridae	Goodchild, 1952; Takanona and Hori, 1974
	Reduviidae	Cohen, 1993
amylase	Miridae	Goodchild, 1952; Takanona and Hori, 1974; Hori, 1975
esterase	Miriade	Goodchild, 1952
galactosidase	Miridae	Hori, 1975
glucosidase	Miridae	Hori, 1975
invertase	Miridae	Goodchild, 1952
lipase	Miridae	Goodchild, 1952
maltase	Miridae	Goodchild, 1952
pectinase	Miridae	Takanona and Hori, 1974

1.3 PROTEINASES

Protein digestion in the Heteroptera has been studied for several reasons. Characterisation of protein digestion contributes to the overall understanding of nutritional processes. Protein digestion in hemipteran insects has also been used in developing theories on the evolution of the Hemiptera. For these reasons, characterising the proteinase activities of the green mirid forms a major focus of this thesis. Protein digestion in the Hemiptera has also been studied for economic reasons. Proteinases constitute part of the secreted salivary enzyme mix believed to cause feeding damage to crops. In addition, new technologies for pest control include the use of transgenic plants expressing genes detrimental to insects. Examples of these are the

genes encoding proteinaceous toxins such as *Bacillus thuringensis* δ-endotoxin (Benedict *et al.*, 1992), and proteinase inhibitors which can increase the resistance of the plant to insect pests (Hilder *et al.*, 1987). A thorough background knowledge of how an insect digests proteins is imperative to fully understanding and optimising these control strategies.

In some literature the term protease is used to describe both endopeptidases and exopeptidases, while the term proteinase refers only to endopeptidases. However in most cases protease and proteinase are used synonomously, and the latter will be used here with the broad definition. There are four mechanistic classes of proteinases, each of which has a characteristic set of functional amino acids which form the active site of the enzyme. The four mechanistic classes are serine proteinases, cysteine proteinases, aspartic proteinases, and metallo-proteinases. These are further divided into six families: serine proteinases I, serine proteinases II, cysteine proteinases, aspartic proteinases, metallo proteinases I and metallo proteinases II (Neurath, 1984). There is one other group of proteinases which warrants definition, the cathepsin proteinases. Most cathepsins are cysteine proteinases (for example cathepsins B and H), but cathepsin proteinases also include serine proteinases (cathepsin G) and aspartic proteinases (cathepsin D). The term cathepsin has varied in its definition in the past, but is generally used to describe endoproteinases from animal tissues, without restriction to acid pH optima (Barrett and Heath, 1972). Cathepsins differ from each other chemically and in their protein sequence. Cathepsin B from mouse, and cathepsins H and L from rat, have about 40% identity in their amino acid sequences (Qian *et al.*, 1991). Cathepsins seem to be more homologous to other cathepsins of the same type in a different species than to different cathepsins within a species, due presumably to gene divergence and functional conservation within subtypes.

Data for determining the class of a proteinase can be obtained in several ways. Chemical modification of catalytic residues can be used to establish mechanistic class, but this can be difficult to interpret due to the possibility of chemical modification of non-catalytic sites (Dunn, 1989). Different proteinase classes have different pH optima, so this can also be used to help categorise proteinases. However the pH ranges

of the different classes are quite broad and can overlap, so that this method alone is not sufficient for categorisation. Specific inhibitors and specific substrates are the most common means of determining the class of a proteinase, as they do so based on the catalytic group of the enzyme. Inhibitor and substrate specificity also have been used to characterise specific proteinase types within a class.

Aspartic proteinases, also referred to as carboxyl proteinases, include cathepsins D and E, and pepsin. Neither the aspartic proteinases nor the metallo-proteinases form covalent intermediates with the substrate during catalysis (Dunn, 1989). Instead, aspartic proteinases catalyse peptide bond cleavage via the aspartic acid residues at positions 32 and 215 (based on the porcine numbering system). The pH optimum of this group is betwen 2 and 5 (Barrett, 1977). Metallo-proteinases, as the name suggests, utilise a metal ion, usually zinc, for catalysis. This group includes thermolysin and the carboxypeptidases A and B, and shows optimal activity at pH 7 to pH 9 (Barrett, 1977).

Cysteine and serine proteinases have been more extensively characterised than the previous groups, and are far more commonly found in insect studies. Both these groups comprise enzymes which have nucleophilic amino acids at the catalytic site, and which form covalent complexes with the substrate during catalysis (Dunn, 1989). Cysteine proteinases include mammalian lysosomal cathepsins B, H, L and S, and the plant enzyme papain (Neurath, 1989). The main active site residue of cysteine proteinases is Cys 25, and catalysis is facilitated by the adjacent residues Asp 158 and His 159 (numbering is based on the papain numbering system, Cohen *et al.*, 1986). The thiol group of Cys 25 attaches covalently to the substrate, cleaving the peptide bond and forming a thiol ester intermediate (Neurath, 1989). The pH optimum for this reaction is usually between 4 and 7 (Barrett, 1977). All cysteine proteinases show some conservation of residues around the catalytic cysteine 25.

Serine proteinases include the well characterised trypsin and chymotrypsin, and also elastase, thrombin and pancreatic kallikrein (Neurath, 1989). The active site residues of serine proteinases are Asp 102, Ser 195, and His 57 (numbering is based on the bovine chymotrypsin system). Serine 195 acts in much the same way as Cys 25 of

cysteine proteinases, attaching covalently to the substrate to form an enzyme-substrate intermediate (Neurath, 1989). The pH optimum for this reaction is usually 7 to 9 (Barrett, 1977), with some serine proteinases remaining active even in the extreme alkaline conditions of lepidopteran midguts (Johnston *et al.*, 1995). Substrate specificity of serine proteinases is determined by the size and shape of the substrate binding site. This site forms a pocket close to Ser 195, into which the side chain of the substrate fits (Kraut, 1977). Trypsin is one of the most sepcific of the serine proteinases, and cleaves adjacent to basic amino acids. Thrombin, like trypsin, catalyses arginyl and lysyl bonds, but it is highly specific for certain sites within its substrate fibrinogen. Chymotrypsin preferentially cleaves on the carboxyl side of aromatic amino acids, but will also cleave large hydrophobic residues at a slower rate. Elastases prefer small, uncharged, non-aromatic side chains, but are less specific than either trypsin or chymotrypsin (Kraut, 1977). Different serine proteinases can often be differentiated using specific substrates and inhibitors.

1.4 BIOCHEMICAL CHARACTERISATION OF HEMIPTERAN PROTEINASES

Most studies examining digestive proteinases in heteropteran insects have focussed on seed-feeding, haematophagous or predatory species. These studies have demonstrated the presence of proteinase activity in both the salivary glands and midgut of heteropteran insects. In the salivary glands, alkaline proteinase activity has been frequently detected through the use of general substrate assays performed under alkaline conditions (Goodchild, 1952; Rastogi, 1962; Hori, 1970; Laurema *et al.*, 1985; Cohen, 1990). In the predator *Zelus renardii,* the salivary gland proteinase activity has been further characterised using specific substrates and inhibitors and is a trypsin-like enzyme involved in pre-oral digestion (Cohen, 1993). In addition to an alkaline proteinase, Laurema *et al.* (1985) detected a second proteinase in the salivary glands of *Lygus disponsi*, with optimal activity against hemoglobin at pH 3. This enzyme was proposed to be a lysosomal cathepsin D not involved in digestion.

Both acidic and alkaline proteinases have also been detected in the midgut of heteropteran insects, with acidic proteinases apparently more common (Terra and Ferreira, 1994). Evidence supporting this includes the acidic nature of the guts of insects within the Heteroptera (Terra and Ferreira, 1994). This environment is more suited to cysteine and aspartic proteinases than to serine proteinases, which have optimal activity in alkaline conditions. Furthermore, cysteine proteinase activity, characterised as cathepsin B-like activity, has been observed in the guts of insects from 4 families of Heteroptera (Houseman, 1978; Houseman and Downe, 1982b; Houseman *et al.*, 1984; Houseman *et al.*, 1985). Identification of cathepsin B was based on an acidic pH optimum, specific substrate hydrolysis (of *N*-benzoyl-DL-arginine-*p*-nitroanilide and *N*-benzoyl-DL-arginine napthylamine), inhibition by cysteine proteinase inhibitors (2-iodoacetamide and tosyl-L-lysine chloromethyl ketone), and activation by thiol reagents and ethylenediamine tetraacetic acid. Aspartic proteinases, characterised as cathepsin D-like proteinases, have also been found in the guts of insects from 7 families of Heteroptera (Houseman and Downe, 1982a; Houseman and Downe, 1983). Again, identification was based on an acidic pH optimum, substrate preference, and inhibition by a specific inhibitor (pepstatin).

The predominance of acidic proteinases in the guts of insects within the Heteroptera is in contrast with the serine proteinases, usually trypsin-like, found in the guts of most other insect groups. As an explanation, it has been proposed that the ancestral hemipteran lost serine proteinase activity in the gut due to low levels of protein in the diet (Houseman and Downe, 1983). From this ancestor arose the ancestral Heteroptera which were primarily predaceous (Goodchild, 1966; Cobben, 1978). Houseman and Downe (1983) suggest that upon returning to a predaceous diet, which is high in proteins, the ancestral Heteroptera adapted by secreting previously lysosomal proteinases into the gut to complete protein digestion.

1.5 MOLECULAR GENETIC CHARACTERISATION OF INSECT PROTEINASES

There have been no molecular characterisations of hemipteran digestive proteinases to date. Molecular studies of insect digestive proteinases have mainly focussed on the serine proteinase genes, paricularly trypsin and chymotrypsin, of Diptera and Lepidoptera. Several trypsin genes have been cloned from insects belonging to these orders, including the insects *Aedes aegypti* (Kalhok *et al.*, 1993), *Manduca sexta* (Peterson *et al.*, 1994), *Drosophila melanogaster* (Davis *et al.*, 1985), and *Lucilia cuprina* (Casu *et al.*, 1994). In most cases, digestive trypsin proteinase genes occur as multigene families. For example, at least two, and up to seven, trypsin genes are expressed in the midgut of *Anopheles gambiae* at different times after a blood meal (Müller *et al.*, 1993), and a family of 18 midgut trypsin genes has been cloned from *Helicoverpa armigera* (Gatehouse *et al.*, 1997).

Insect chymotrypsin genes have been studied less extensively than trypsin genes. Single midgut chymotrypsin genes have been cloned from *Plodia interpunctella* (Zhu *et al.*, 1997), *Manduca sexta* (Peterson *et al.*, 1995), and *Aedes aegypti* (Jiang *et al.*, 1997). The *A. aedes* chymotrypsin, like the trypsin gene, is induced after a blood meal. In addition, a family of 14 chymotrypsin genes has been cloned from *Helicoverpa armigera* (Gatehouse *et al.*, 1997). Insect serine proteinases have the same catalytic residues as the vertebrate proteinases (Asp 102, Ser 195, and His 57), and show similar levels of conservation between genes.

Molecular studies of insect cysteine proteinases are also limited. A procathepsin L-like gene, involved in differentiation of imaginal discs, has been cloned from *Sarcophaga peregrina* (Homma *et al.*, 1994). Cathepsin L-like genes have also been cloned from a haemocyte cell line of *D. melanogaster* (Tryselius and Hultmark, 1997) and from the eggs of *Bombyx mori* (Yamamoto *et al.*, 1994). Four cathepsin L-like genes have been cloned from the maize weevil *Sitophilus zeamais* (Matsumoto *et al.*, 1997), but only one has been characterised, and this is expressed in several tissues including the gut caecae, but not in the midgut. The only midgut specific cysteine

proteinase cloned from insects to date is from *D. melanogaster* (Matsumoto *et al.*, 1995) and this gene has homology with both cathepsin H and cathepsin L genes.

1.6 THESIS OUTLINE

The aim of this thesis is to perform initial characterisation of the digestive system of the green mirid, *Creontiades dilutus* (Fig. 1.8). The green mirid belongs to the family Miridae of the infraorder Cimicomorpha, and is a sucking pest of several Australian agricultural crops, including cotton and lucerne. Mirids go through 5 or 6 nymphal stages before becoming adults of approximately 8 mm in length, with the entire life cycle taking around 4 weeks (Carver *et al.*, 1970).

Digestive system structure varies enormously within the Hemiptera, making it dangerous to extrapolate information obtained from one species to another. Also, only a relatively small number of studies of the morphology of the digestive systems of the Hemiptera have been performed, and none of these looked at the green mirid specifically. Investigation of the structure of the digestive system of the green mirid is an important first step to characterising the digestive processes, and to understanding the digestive physiology and biochemistry at a functional level. For these reasons a morphological examination of the green mirid digestive system was performed. Although biochemical characterisations of hemipteran proteinase activity are more common than morphological studies, they have generally focussed on blood feeding and predatory Heteroptera. Examination of the proteinases of the green mirid, as a primarily phytophagous heteropteran, has the potential to offer new perspectives to this field and forms the second section of this thesis. Characterisation of digestive proteinases is not only beneficial to understanding digestive physiology, but also has the potential to provide information on the evolution of the Hemiptera. Finally, there is a notable absence of molecular genetic studies of proteinases from hemipteran insects which would complement the biochemical studies and provide a more complete picture of hemipteran protein digestion. Thus an examination of proteinase genes from the green mirid forms the third section of this thesis.

Figure 1.8: The green mirid, *Creontiades dilutus*

CHAPTER 2:
STRUCTURE OF THE DIGESTIVE SYSTEM OF THE GREEN MIRID, *CREONTIADES DILUTUS.*

2.1 INTRODUCTION

The insect digestive system is a highly integrated system, the various functions of which are reflected by its morphology and by that of its constituent cells. The salivary glands and the gut, comprised of the foregut, midgut and hindgut, together form the major functional organs of the insect digestive system. The salivary glands play a role in the production of digestive enzymes, the foregut is primarily a storage organ, and the midgut is the main site of digestion and absorption of food. The hindgut typically functions as part of the excretory system and was excluded from the following study.

There are two main areas of interest in hemipteran salivary glands beyond the quest for basic knowledge. Firstly, salivary gland structure is a useful tool in the classification of hemipteran insects (Southwood, 1955). Secondly, for economic reasons there have been several studies examining the ability of saliva to damage plants through both the enzymes present, and the capacity for virus transmission through the saliva (Gibb and Randles, 1989). The structure of the salivary glands of the Cimicomorpha has been discussed in Chapter 1. Briefly, each salivary gland consists of an accessory gland and a larger principal gland, which is divided into an anterior and a posterior lobe.

Although ultrastructure can vary among species, the cells of the salivary glands of heteropteran insects are usually binucleate secretory cells, which Baptist suggested can vary in structure according to the insect's food source (Baptist, 1941). For example, principal glands of carnivorous Heteroptera were observed to have a small lumen surrounded by long columnar cells, while in herbivorous Heteroptera the cells are cuboidal and the lumen is larger (Bronskill *et al.*, 1958). The cell structure can also differ markedly in the different lobes of the principal gland within a species (Breakey, 1936; Hori, 1967). In addition, a notable difference in histological staining has been

reported for the lumens of the anterior and posterior lobes of the principal gland of some mirids (Goodchild, 1952). The difference in cell structure and lumen content implies that the anterior and posterior lobes of the principal gland perform different functions in heteropteran insects. As mentioned in Chapter 1, it has been proposed that the anterior lobe produces salivary sheath material or a watery lubricating solution, and that the posterior lobe is the site of digestive enzyme production (Miles, 1967).

Most of what is known about the morphology of the gut of the Heteroptera is based on a few large studies (Breakey, 1936; Goodchild, 1952; Goodchild, 1963; Southwood, 1955), which have demonstrated extensive variation in gut structure within this group. The midgut of cimicomorphan insects is simple, consisting of an anterior sac-like region and a posterior tubular region which is further divided into two sections by a constriction in some subfamilies (Goodchild, 1952). The different regions of the midgut often perform different functions. In some blood and seed-feeders, the anterior midgut functions primarily as a storage organ and digestion occurs in the posterior midgut (Terra *et al.*, 1988; Silva and Terra, 1994), while in predators digestion occurs along the length of the midgut, although the enzyme content of the different regions can vary (Cohen, 1993). The functions of the different regions of the midgut in plant cell-feeding heteropterans is unknown.

The cells of the heteropteran midgut in general are mostly binucleate, although there is usually a proportion of uninulceate cells also present. The gross structure of the cells of both the anterior and posterior midgut of heteropterans appear to be similar, with a narrow base and a bulbous tip which projects into the lumen, although the shape of the cells can vary significantly with the amount of distension of the gut (Goodchild, 1952; Billingsley and Downe, 1983). In the phytophagous families Tingidae and Miridae, a second cell type, possibly involved in absorption, has been observed in the tubular posterior midgut, with a more cuboidal base and a long, thin lobe projecting into the lumen (Goodchild, 1963).

In several heteropteran insects the midgut cells are ensheathed by a second membrane, the perimicrovillar membrane, and covered by multiple extracellular membrane layers (ECML) (Lane and Harrison, 1979; Billingsley and Downe, 1988;

Silva *et al.*, 1995). These membranes appear more extensive in the posterior than in the anterior midgut in *R. prolixus* (Billingsley and Downe, 1989), which is also the site of the majority of digestion in these insects. In addition, the ECML can be associated with digestive enzymes, supporting a role in the compartmentalisation of digestion (Ferreira *et al.*, 1988).

The aim of the work in this chapter is to examine the structure and some physiological characteristics, such as the pH, of the digestive system of the green mirid, to provide a framework for interpreting the biochemical and molecular biological studies presented in subsequent chapters. The specific structural questions being addressed include whether the anterior and posterior lobes of the principal salivary gland, or the anterior and posterior regions of the midgut, show any differences that might reflect functional differences within these organs.

2.2 MATERIALS AND METHODS

2.2.1 Insect Rearing

Insects were collected every one to three weeks from lucerne crops (*Medicago sativa* cvs. Aurora and L69) near Canberra, Australia, over the months of November through March. Nets were used to sweep lucerne fields, and a battery operated aspirator was used to select the green mirids. Mirids were kept in the laboratory in a 27 cm × 38 cm × 45 cm mesh cage, and fed lettuce, round beans and sprouting potatoes. Every second day fresh food was provided, and the old lettuce discarded. The old beans and potatoes contained any eggs which had been laid, and were put in a 21.5 cm × 13 cm × 9.5 cm mesh covered box in a constant temperature room (25°C, 40% humidity, 14 hours light) to allow the eggs to hatch. After one week, lettuce and green beans were provided for the emerging nymphs, and this food was also changed every second day. The nymphs were kept in these boxes until the 5th or 6th instar, and then were put into a cage containing the laboratory population.

2.2.2 Dissections

Insects were immobilised either by exposure to CO_2 or by chilling on ice before being dissected. Dissections of salivary glands and guts were performed under cold insect saline solution (0.1 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $NaHCO_3$, pH 7) under 120x magnification using a dissecting microscope (Olympus, model number SZ4045). The insect was placed dorsal side down, and the head was removed by pulling with forceps. By continuing to pull slowly and continuously, the salivary glands were removed from the thorax. The salivary ducts were cut close to the head using a scalpel to separate the entire salivary gland complex. The alimentary canal was also cut as close to the head as possible, in the oesophageal region, using a scalpel. The entire gut was then removed by slowly pulling from the posterior of the abdomen, and was then severed from the anus. Occasionally, the abdominal cuticle was slit to aid in this extraction.

2.2.3 Microscopy

Dissected tissues were immediately fixed in 2.5% gluteraldehyde in washing buffer (0.1 M PO_4, 0.15 M sucrose) for at least one hour. Fixed tissues were washed three times in washing buffer for a total of at least one hour, then left rotating at room temperature for one hour in 1% osmium tetroxide (OsO_4) in washing buffer. Tissues were dehydrated serially through an ascending series of alcohol (30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol and 100% ethanol) for at least 30 min in each concentration. Tissues were then infiltrated with LR White acrylic resin (medium grade, The London Resin Company Ltd.) by incubating in increasing proportions of resin (1 resin/2 ethanol, 2 resin/1 ethanol) for at least 30 min, and finally in pure resin overnight. Specimens were then embedded in gelatin capsules containing fresh resin and polymerised at 60°C overnight.

For light microscopy, 0.5 µm sections were cut and stained with toluidine blue. For ultrastructural analyses, transmission electron microscopy (TEM) 0.05 µm sections were cut and examined with a Hitachi 600 or Hitachi 7100 at 75 kV.

2.2.4 Feeding Experiments

2.2.4.1 Dyes

Dyes were used to examine both the porosity of the gut to charged molecules, and the path of ingested material. Rose Bengal (George T. Gurr Ltd., England), an acidic dye 1.05 kDa in size, was diluted to 2% in 15% sucrose. Nile Blue sulphate (Allied Chemical, N.J.), a basic dye 0.733 kDa in size, was diluted to 1% in 15% sucrose. Each of the two dyes, plus a 1:1 mixture of the two, were sandwiched between two sheets of Parafilm® (American National Can™, Chicago) and stretched over a plastic petri dish. Ten mirids were left to feed overnight on each of these solutions. The following morning insects were cooled and dissected tissues were examined by light microscopy (Olympus, model number SZ4045) to determine the location of the ingested dye.

2.2.4.2 Horse Radish Peroxidase

Horse radish peroxidase (HRP) (Sigma Chemical Co., St Louis, Missouri) is a protein with a molecular weight of 44 kDa that was used to examine the porosity of the mirid gut to larger molecules. On each of six days, six adult mirids and six third instar nymphs were removed at random from the laboratory population. From these, three adults and three nymphs were placed in a plastic vial covered with Parafilm® and fed 7.5% sucrose. The remaining three adults and three nymphs were placed in a plastic vial covered with Parafilm® and fed on a solution containing 7.5% sucrose and 2.5 mg/ml HRP. Mirids were left to feed overnight, then dissected and assayed as follows to determine the location of the HRP in the digestive system.

The mirids were dissected in random order with respect to age and feeding treatment. Haemolymph was extracted from each mirid by wounding a leg and collecting the exudate into a capillary tube. Approximately equal volumes of haemolymph from three mirids of the same age and feeding treatment were combined in microfuge tubes and diluted to 80 µl with distilled water. The salivary glands, midgut and Malpighian tubules were also collected from each mirid. Keeping each tissue type separate, samples from the three mirids within each age and feeding type were combined in microfuge tubes and ground in 80 µl of distilled water. These samples were then centrifuged at 8000 *g* for 5 minutes and the supernatants recovered.

HRP activity was assayed by combining 50 µl of each sample with 50 µl of a solution of 0.5 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.1 M citric acid, pH 4.2, in a 96 well assay plate, and reading the absorbance at 405 nm after 15 minutes. Analysis of Variance (ANOVA) tests were used to compare the mean amount of peroxidase activity in the samples, and were performed using Genstat 5, Release 3.1. Data were transformed using natural logarithms to meet normality criterion prior to ANOVA testing.

2.2.5 pH

Mirids were placed in mesh covered boxes for 16 - 20 hours and given water, round beans and lettuce (fed mirids), or water only (starved mirids). Universal Indicator (BDH Ltd., England) was used to determine the pH of the salivary glands and midgut of fed and starved mirids. Salivary glands and midguts were dissected in insect saline solution and rinsed in distilled water. Midgut luminal contents were flushed from the midguts by gentle teasing and collected with a capillary tube. Salivary glands and midguts from individual mirids were crushed separately in 10 to 20 µl of 20% Universal Indicator. Midgut contents were also mixed with 10 to 20 µl of 20% Universal Indicator. Colour change was observed visually relative to standards of known pH. Samples from at least 17 mirids were tested, and t-tests (calculated using StatView® SE+Graphics) were used to compare the means of the pH values.

2.3 RESULTS AND DISCUSSION

Most studies examining the structure of heteropteran digestive systems in detail are at least 30 years old, with nearly all recent studies focussing on the hematophagous reduviid *Rhodnius prolixus*. Because of this, and also because of the descriptive nature of the work, references to the literature have been included where relevant in the following combined Results and Discussion.

2.3.1 Salivary Glands

2.3.1.1 General Morphology

The salivary glands of the green mirid are paired and lie in the thoracic region of the insect. They are quite large, extending for almost the entire length of the anterior midgut (Fig. 2.1). On each side of the gut is a principal gland, an accessory gland, a principal duct and an accessory duct. The principal gland is bilobed, with the anterior and posterior lobes being of roughly equal size and joining at the hilus. The anterior and posterior lobes differ in external appearance, with the posterior lobe being more folded than the anterior lobe. The accessory gland is kidney shaped and smaller than the principal gland, and sits at the posterior end of the posterior lobe of the principal gland apparently attached to the midgut. No differences were observed in the structure of the salivary glands at the different stages of development in the mirid. The large size of the salivary glands of the green mirid imply an important role for them in feeding and/or digestion.

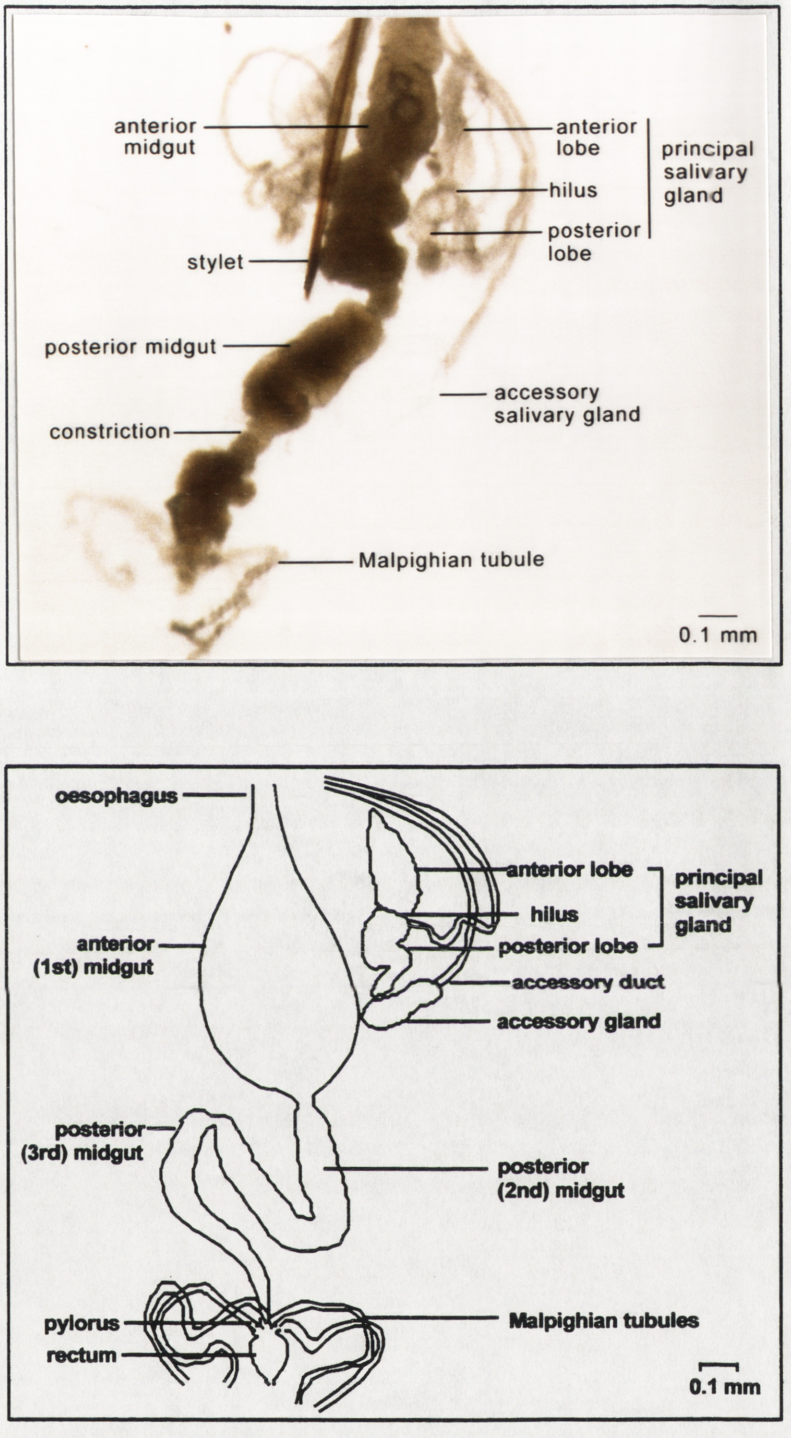


Figure 2.1: Alimentary canal and salivary glands of the green mirid. **A)** Photograph of midgut region and salivary glands. Note hindgut is not included. **B)** Sketch of entire gut and salivary glands. Note only one of the pair of salivary glands is shown. Bracketed numbering of midgut sections corresponds to regions identified by Goodchild (1952).

2.3.1.2 Microscopy

The cells of both the anterior and posterior lobes of the principal salivary gland appear to be mostly uninucleate, and contain abundant secretory vesicles, presumably for the production of the saliva. The cells of the posterior lobe are approximately 1.5 times larger than those of the anterior lobe, and are slightly more columnar in shape (Fig. 2.2). In the anterior lobe the infolding of the cell membrane in the basal region appears more extensive than in the posterior lobe and the nuclei are apparently larger, at least as a proportion of the cell size itself (Fig. 2.2 A). Significantly, the lumen contents of the two lobes stained differently with toluidine blue stain. The contents of the anterior lumen are very granular and darkly stained, while the contents of the posterior lumen are paler and less granular, appearing more uniform in staining. The marked difference in cell structures, and the apparently different luminal fluids, suggest that the anterior and posterior lobes of the principal salivary gland serve different functions in the green mirid.

2.3.2 Midgut

2.3.2.1 General Morphology

The gut of the green mirid is similar to that described for cimicomorphan insects by Goodchild (1966) (Fig. 2.1). There is only a short oesophageal region, and no obvious foregut. Gastric caecae are absent, as they are for all Heteroptera. The midgut is tubular, and comprises an anterior sac-like region and a longer, narrow posterior tubular region. The tubular region is further divided slightly posterior to the middle of its length by a constriction. This constriction presumably corresponds to that seen in the Bryocorinae (Goodchild, 1952). The anterior midgut can vary in size from being quite small and deflated to being very distended, with either food or air bubbles, forming a large sac. The presence of air bubbles may aid moulting, or may be a normal condition in the mirid, for example during starvation. The posterior midgut is of a fairly constant width, except for the narrowing at the constriction mentioned above. It is folded into a loop inside the abdomen, bending once to pass forward, and bending

again to continue posteriorly towards the rectum (Fig. 2.1 B). Strong peristaltic movements were often observed in both the anterior and posterior midgut.

The four Malpighian tubules join the extreme posterior midgut at the pylorus (Fig. 2.1). The pyloric region itself is not large. There is a slightly enlarged region where each Malpighian tubule joins the gut, but it is unknown whether this is an enlargement of the tubule or of the pylorus. The pyloric region joins the hindgut, which is short, sac-like, and has no obvious separate ileum and rectum.

The infraorders Cimicomorpha and Pentatomomorpha are believed to have split early in the evolution of the Heteroptera, as zoophagous and phytophagous groups respectively (Goodchild, 1963). Although primarily a plant feeder, the gross morphology of the gut of the green mirid more closely resembles that of predatory Cimicomorpha than it does the guts of the plant feeding Pentatomomorpha (see Fig. 1.5). In other words, the taxonomy of the mirid is a better predictor of the structure of the gut than are the dietary habits, which is consistent with insect gut morphology in general (Terra and Ferreira, 1994).

2.3.2.2 Microscopy

The cells lining the midgut of the green mirid appear to be of one structural type. This situation is more consistent with the description of zoophagous cimicomorphs than with phytophagous ones (Goodchild, 1963), although the presence of a second cell type cannot be ruled out since every cell of the midgut was not examined. The midgut cells appear similar in structure to those described previously for heteropteran insects (Goodchild, 1963), being large, modified columnar cells, with bulbous tips protruding into the lumen of the gut (Fig. 2.3). The cells were mostly uninucleate, although some binucleate cells were also observed. The luminal border of the midgut cells is lined by a brush border, which appears as a thick, uniform layer under light microscopy (Fig. 2.3). The basal region of the midgut cells appears very infolded, probably to increase the surface area for transportation between the gut cells and the haemolymph. Basal infoldings can also be involved in concentrating solutes to create an osmotic pressure gradient for water absorption. The amount of infolding may

be related to feeding, as in *R. prolixus* where the basal labyrinth separates out after a blood meal (Billingsley and Downe, 1983), but further studies of guts at different stages of feeding are required to determine this.

The cell structure does not differ grossly between the anterior and posterior midgut, although some minor differences were observed. Near the apical borders the cells are tightly joined, but towards the basal ends large spaces between the cells were often observed. Such intercellular spaces are common between cells which are actively secreting, often receiving material from the secretory vesicles, and so are usually associated with cells which possess abundant vacuoles (Raes and Verbeke, 1994). This is consistent with observations in the green mirid, where the intercellular spaces are more extensive, and the vacuoles are more abundant, in the anterior region of the midgut than in the posterior segment (Fig. 2.3). Overall, an extremely large number of vacuoles are present in the midgut cells, possibly containing storage material such as lipids, secretory material such as digestive enzymes, or material which either neutralises salivary enzymes or enhances their activity, for example by controlling the pH of midgut. Alternatively, the vacuoles could contain digestive products absorbed by the cell .

Electron microscopy confirmed that the anterior and posterior midgut cells were structurally very similar, with some minor ultrastructural differences that mainly resulted from the anterior cells possessing more storage and secretory vesicles. Specifically, large aggregates of lucent storage areas, possibly containing secretory proteins or poorly stained lipids, were observed in the anterior cells (Fig. 2.4 A). Similar storage droplets were occasionally seen in posterior cells, but here they were isolated rather than aggregated and were far less abundant. In *R. prolixus* large storage inclusions, usually containing lipids, are also common in the anterior midgut cells (Billingsley and Downe, 1989). Since the anterior midgut is the site of food storage and not of digestion in hematophagous Heteroptera, the lipids must be either taken directly into the cells by pinocytosis in these insects, or transported from the posterior midgut via the hemolymph for long term storage (Billingsley and Downe, 1989). In many other heteropteran insects however, digestion apparently does occur in the

anterior midgut (Silva and Terra, 1994; Cohen, 1993), so that the storage inclusions observed in the green mirid may contain digested material absorbed directly from the anterior midgut.

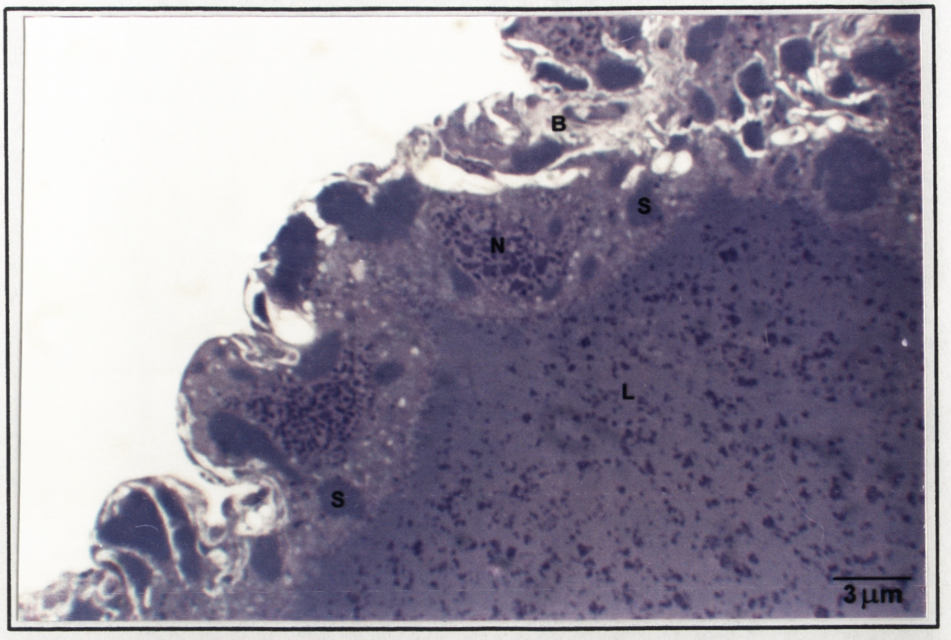
Concretions or spherites with electron dense concentric layers were also observed exclusively in the anterior cells (Fig. 2.4 B). Similar spherites were observed in the anterior midgut cells of *R. prolixus* (Billingsley and Downe, 1983), and may be involved in either the temporary storage of, or the storage and excretion of, salts such as phosphates, chlorides and carbonates. By storing ions, the spherites may play a role in regulating the internal environment of the insect and in its detoxification systems (Martoja and Ballan-Dufrancais, 1984). Membrane bound vesicles with dense contents were also found only in the anterior cells, where they were concentrated at the apex (Fig. 2.4 B). In the seed sucker *Dysdercus peruvianus* (Pentatomomorpha: Pyrrhocoridae), double membrane vesicles observed at the apex of midgut cells were suggested to function in the transport of secretory proteins from the Golgi areas for release into the lumen (Silva *et al.*, 1995). While it was not possible to confirm the presence of a double membrane at the resolution examined, the membrane bound vesicles observed in the green mirid may also be involved in protein secretion.

All midgut cells were lined by a brush border of microvilli which contain slender fibrils that continue into the cytoplasm of the cell (Fig. 2.5). Several membranous layers, extending approximately 0.12 μm - 0.26 μm into the midgut lumen, were commonly seen covering the microvilli of the cells, most extensively in the posterior midgut (Fig. 2.5). These membranes, termed the extracellular membrane layers (ECML) have been previously described in hemipteran insects, particularly in *Rhodnius prolixus*, and have been implicated in performing some of the functions of the absent peritrophic membrane, such as compartmentalising digestion (Lane and Harrison, 1979; Billingsley and Downe, 1989; Werner *et al.*, 1991; Silva *et al.*, 1995). Discontinuously along the luminal border of the cells, several layers of the ECML were stacked so closely as to become an amorphous and very thick lining (Fig. 2.5). The significance of this lining in the green mirid is currently unknown, but it may also contribute to functionally replacing the peritrophic membrane.

Abundant mitochondria were concentrated at the apical end of the cells of both the anterior and posterior midgut (Fig. 2.4 and Fig. 2.5), indicating highly active cells. Concentrations of mitochondria were observed in the apical and basal portions of the midgut cells of *R. prolixus* (Billingsley and Downe, 1983). The abundant mitochondria are presumably required at the ends of the cell to fuel the secretion of digestive enzymes, the absorption of the products of digestion, and transport between the midgut cells and the haemolymph.

Beyond the cells, the lumen of the anterior midgut contained large numbers of protozoans (Fig. 2.4 A), most of which were biflagellate and may belong to the Genus *Retortamonas,* which are common parasites of animal intestines.

A)



B)

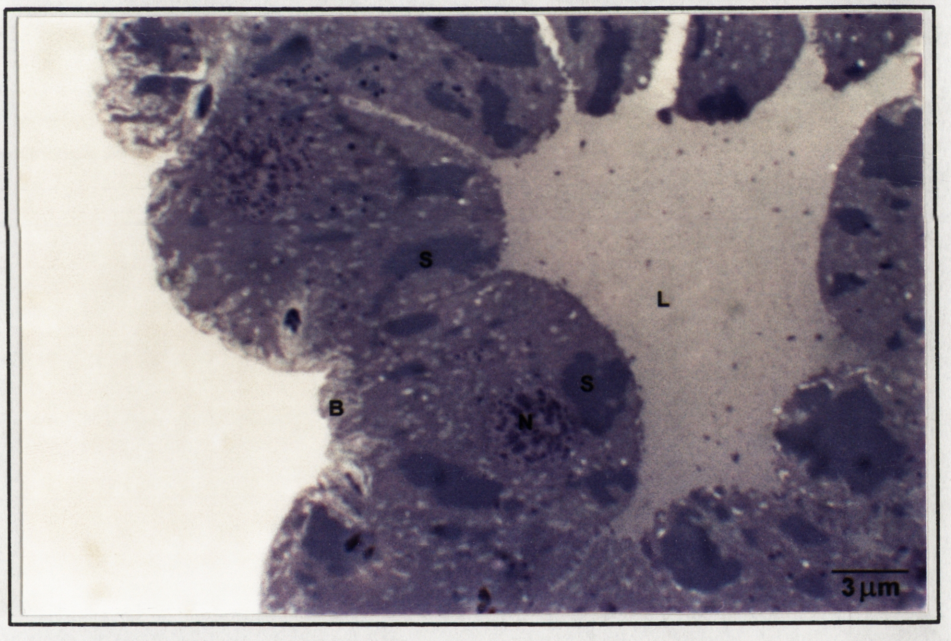


Figure 2.2: Thick sections (0.5 microns) of the principal salivary gland of the green mirid **A)** Anterior lobe and **B)** Posterior lobe.

B, basal region infolding; L, lumen; N, nucleus; S, secretory vesicle

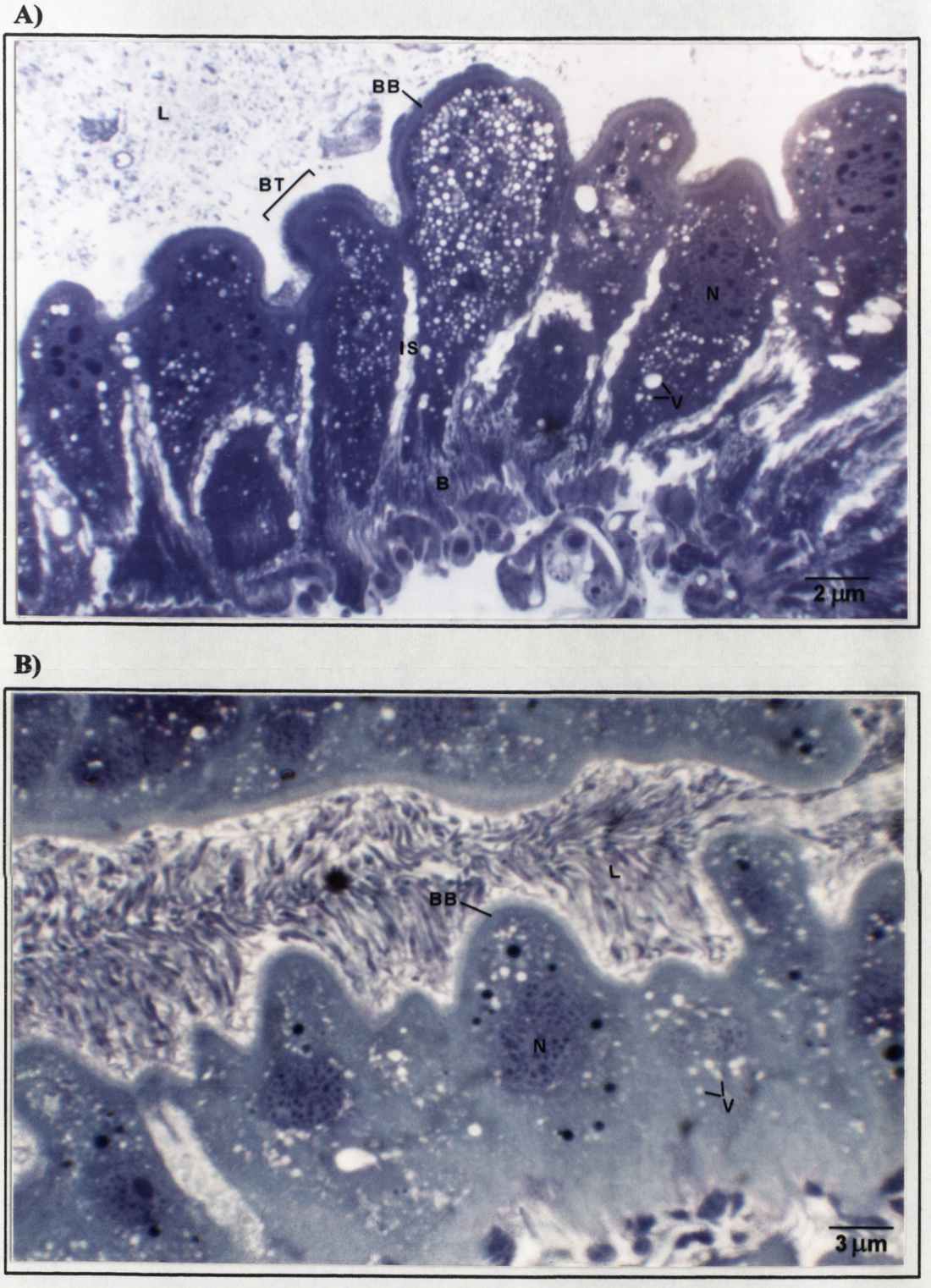


Figure 2.3: Thick sections (0.5 microns) of the midgut of the green mirid, **A)** Anterior midgut and **B)** Posterior midgut.

B, basal region infolding; BB, brush border; BT, bulbous tip; L, lumen; N, nucleus; I, intercellular spaces; V, vacoule

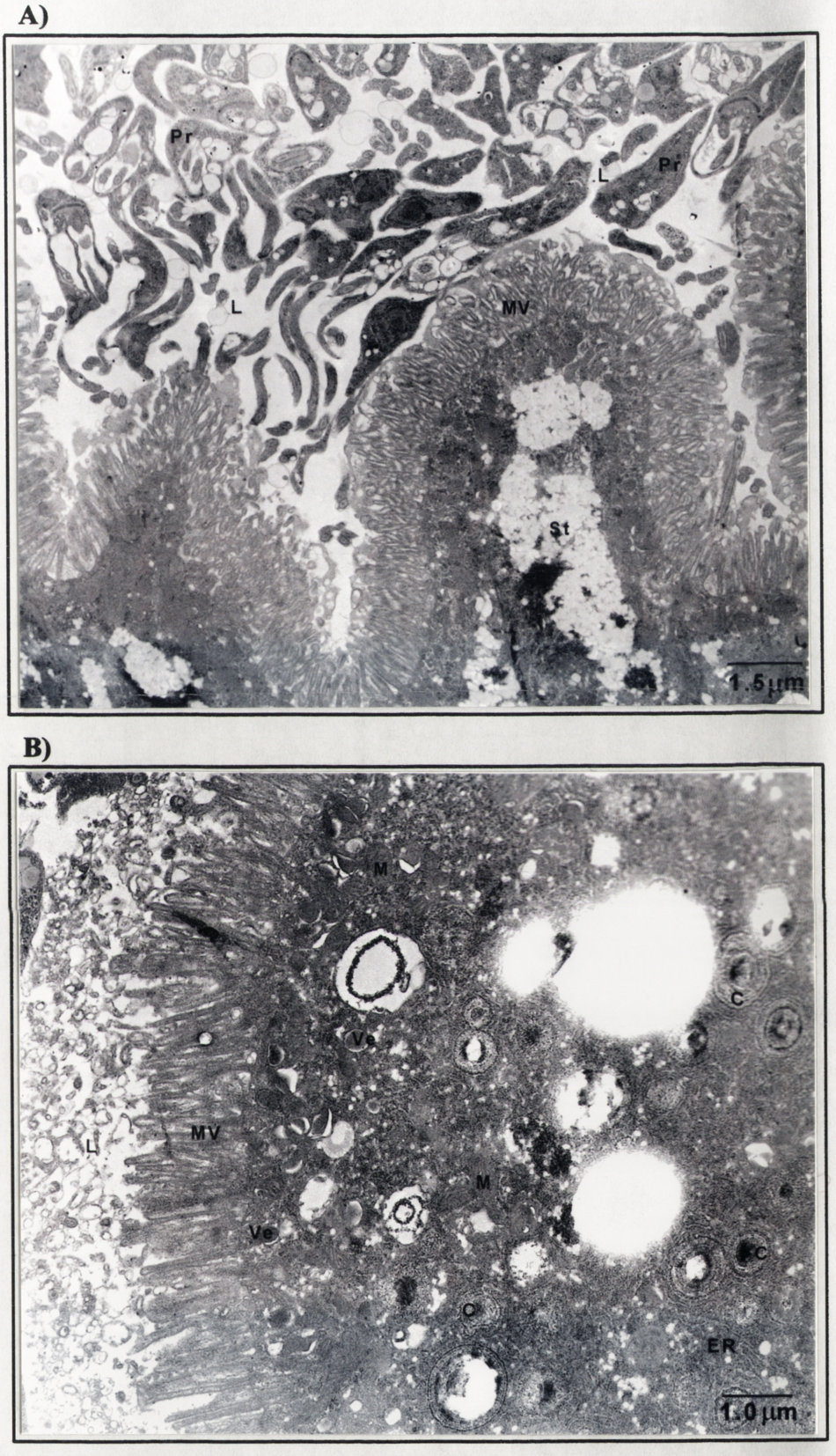


Figure 2.4: **A)** Transmission electron microscopy (TEM) of green mirid anterior midgut,and **B)** TEM of green mirid anterior midgut at a higher magnification.
C, concretion; ER, rough endoplasmic reticulum; L, lumen; M, mitochondria; MV, microvilli; Pr, protozoan; St, storage droplets; Ve, membrane vesicles.

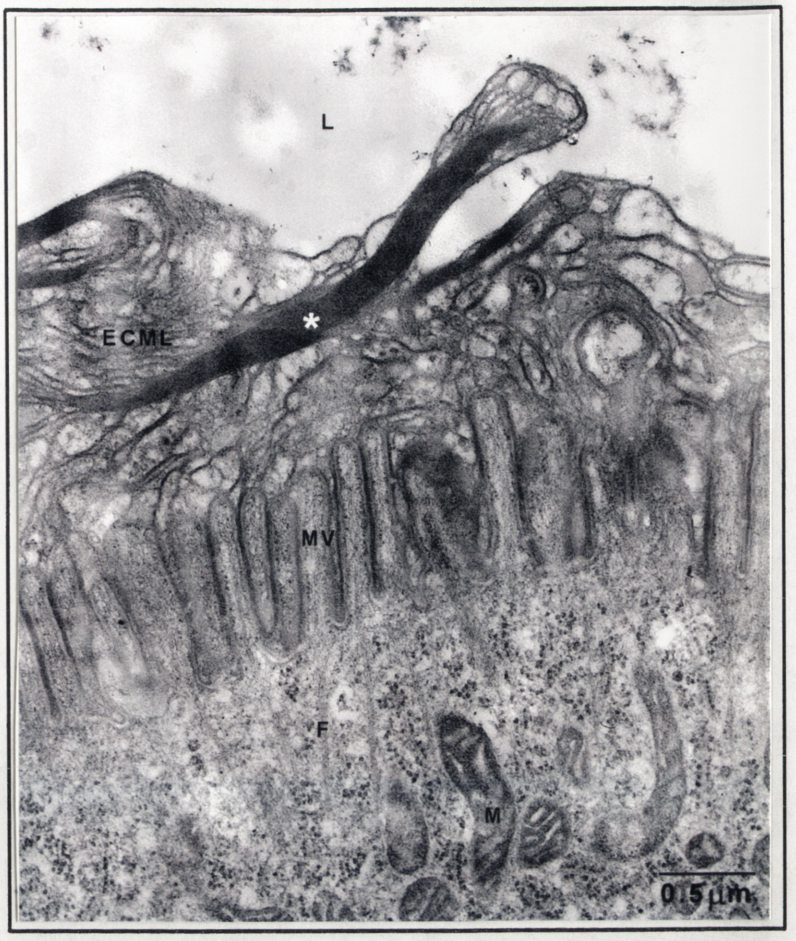


Figure 2.5: Transmission electron microscopy of green mirid posterior midgut. ECML, extracellular membrane layers; F, fibrils; L, lumen; M, mitochondria; MV, microvilli; * stacked membranes (discussed in text)

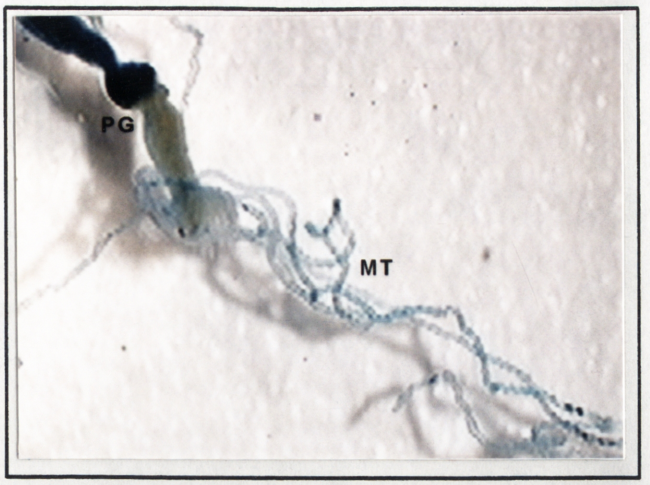
2.3.3 Feeding Experiments

2.3.3.1 Dyes

Mirids which had fed overnight on either nile blue or rose bengal dyes, or a mixture of the two, were dissected to determine the location of the dye. Specifically, to ascertain whether the dye had been absorbed from the gut lumen into the insect haemolymph and then into the Malpighian tubules for excretion. The guts of mirids fed nile blue were obviously blue, confirming the insects had been feeding. In less than half of the dissected mirids which had been feeding (3 out of 10), there was faint blue colour observed in the Malpighian tubules (Fig. 2.6 A). In these insects there were regions of more concentrated colour within the tubules, particularly at the proximal end. The guts of the mirids fed rose bengal were slightly pink, again confirming they had been feeding. The pink colour was very strong in the Malpighian tubules of all mirids which had fed on rose bengal (Fig. 2.6 B). In a few samples the colour was only present in the distal region of the Malpighian tubules, and in a few others the colour was present in the tubules before the dye had travelled to the posterior of the midgut. These two observations make it unlikely that the colour observed in the Malpighian tubules resulted from any back-flow from the gut. The presence of dye in the Malpighian tubules before it had reached the posterior midgut also suggests absorption is occurring in the anterior midgut.

Both acidic and basic dyes of at least 0.733 kDa in size can therefore cross the gut of the green mirid, and can enter the Malpighian tubules presumably via the haemolypmh. However, based on the frequency and intensity of colour, it appears that the rose bengal either more readily crossed the gut, or was more readily absorbed by the Malpighian tubules, than the nile blue. When mixed together in equal amounts, the two dyes appeared very blue in colour. When fed on this mixture, the guts of the mirids also appeared blue in colour. Interestingly though, the colour in the Malpighian tubules was predominantly pink (Fig. 2.6 C). Again this implies the rose bengal is more readily transported through the green mirid. Since rose bengal is slightly larger in size than nile blue, any selection occuring is more likely to be based on the charge of the molecule. This selection could be occurring at either the gut or Malpighian tubule cells, however the Malpighian tubule cells are the most likely site of selectivity as insect Malpighian tubules are known to concentrate acidic dyes (Maddrell *et al.*, 1974).

A)



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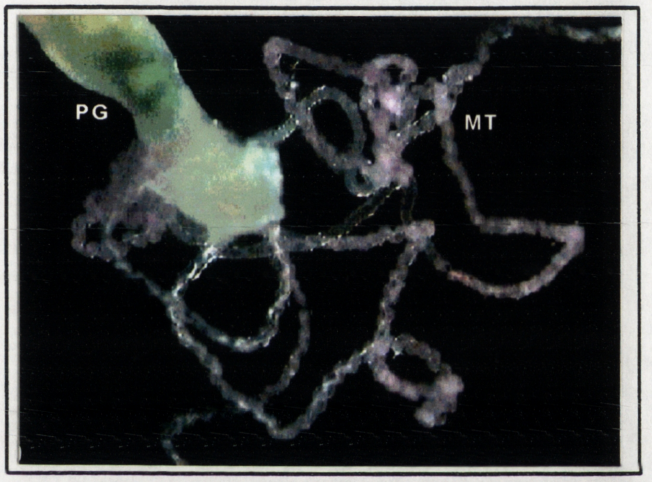

C)

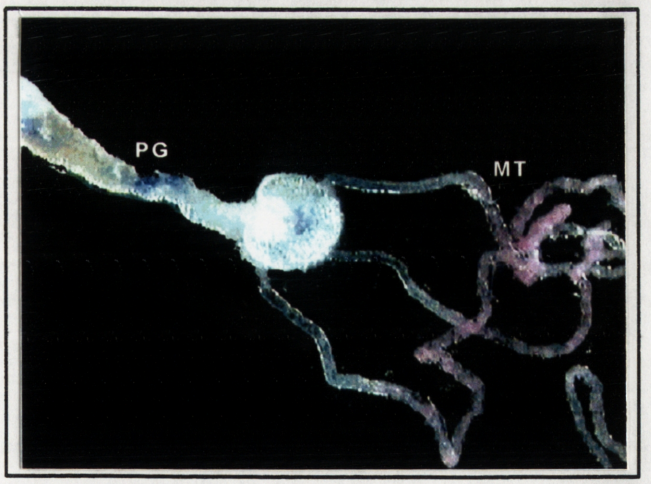

Figure 2.6: Dye feeding experiment. Posterior midgut and Malpighian tubules, dissected in insect saline solution, from mirids fed overnight on 15% sucrose containing **A)** 1% nile blue (mag. x 12) **B)** 2% rose bengal (mag. x 30), and **C)** 1% rose bengal / 1% nile blue (mag. x 20).
MT, Malpighian tubules; PG, posterior midgut

2.3.3.2 Horse Radish Peroxidase

Mirids which had been feeding overnight on horse radish peroxidase (HRP), and mirids which had been fed only sucrose, were dissected to collect haemolymph, salivary gland, midgut and Malpighian tubule samples which were then assayed for HRP activity. The raw data and ANOVA table are given in Appendix 1. The results showed a significant increase in HRP activity in all samples from mirids fed HRP when compared with those fed only sucrose ($F_{1,15}$=92.7; $p<0.001$) (Fig. 2.7). Note that the results from the midgut are uninformative regarding transport across the gut, and demonstrate only that the insects had been feeding. Hence the midgut absorbance values were extremely high and were plotted on a separate axis to all other tissues on Fig. 2.7. The results show that protein molecules of at least 44 kDa in size can cross the gut, enter the haemolymph and pass into the Malpighian tubules intact, without losing biological activity. The presence of large intercellular spaces in the gut may be involved in this porosity, although it is also possible that there are intracellular pathways for the passage of proteins.

The increase in HRP detected in the salivary glands could be due to the haemolymph around the tissue not being rinsed completely. Alternatively, the salivary glands could be absorbing the HRP from the haemolymph. The accessory salivary gland of hemipterans is known to produce a dilute secretion for flushing out food or for excreting excess water. This secretion is a dilute filtrate of the haemolymph, thus the accessory gland functions at least in part as a diuretic organ (Miles, 1972). Miles (1967) also demonstrated that glucose, glycerol and amino acids appear in the watery saliva after injection into the haemolymph of plant bugs from the families Lygaeidae and Pentatomidae. In addition, horse radish peroxidase injected into the haemolymph of *Eumecopus punctiventris* (Pentatomidae) was quickly detected in the accessory salivary gland and the secreted saliva (Miles and Sloviak, 1970). Miles suggested the accessory gland may have an excretory function, removing waste from the haemolymph via the saliva (Miles, 1972). This may in part explain the uptake of HRP by the salivary glands observed in the green mirid.

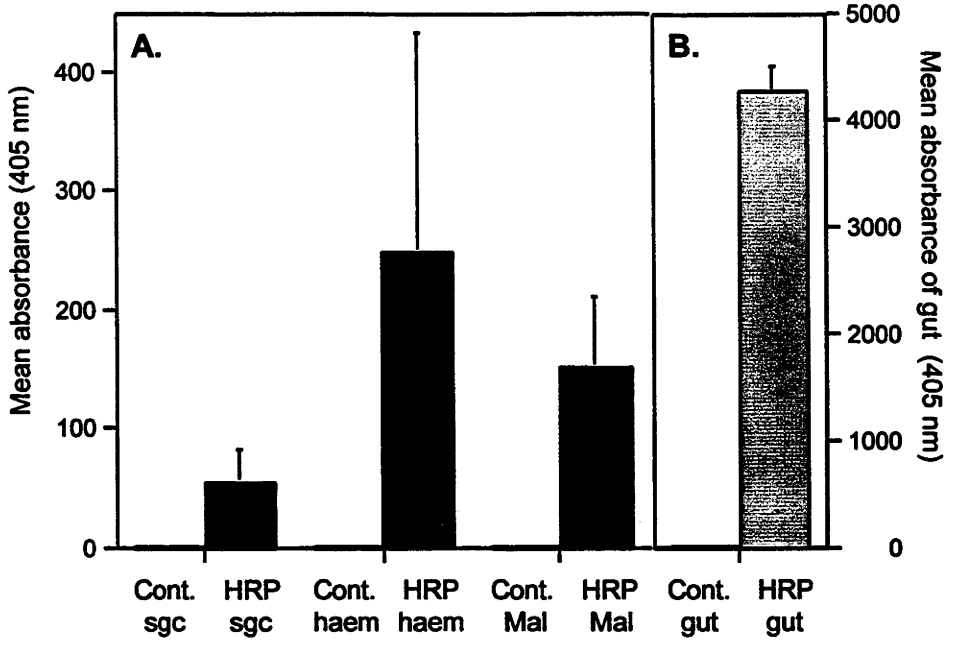

Figure 2.7: Horse radish peroxidase (HRP) feeding experiment. Data shown is the mean absorbance (405nm), ± standard error (SE), measured in samples from either sucrose fed mirids (Cont.) or mirids fed on HRP. Tissues sampled were A. salivary glands (sgc), haemolymph (haem), and Malpighian tubules (Mal.), plotted against the left axis, and B. midgut (gut), plotted against a separate axis to all other samples, shown on the right, due to the uninformative and very high value (see text, section 2.3.3.2).

2.3.4 pH

The pH values calculated for the salivary glands from 17 mirids, flushed midguts from 18 mirids, and midgut luminal contents from the guts of 21 mirids, are given in Table 2.1. There was no difference in pH between salivary glands from fed and starved insects (t_{20}=0.0088; p=0.99) or between midguts from fed and starved insects (t_{25}=0.85; p=0.40). The salivary gland and flushed midgut samples were all of almost neutral pH. The midgut contents however had a lower pH than either the salivary glands or midgut tissue ($t_{>36}$>6; p<0.001), with a slightly acidic pH. This is consistent with the fact that all other hemipteran insects studied have acidic midgut lumens (Terra and Ferreira, 1994), and indicates some acidic secretion, probably into the anterior midgut, is occurring. Alternatively, the process of digestion itself or the

action of bacteria could result in the observed acidic environment. Measurement of the pH of the different regions of the midgut, and at different stages of digestion, may help resolve this.

Table 2.1: pH of the digestive system of the green mirid, ± SE.

Tissue	pH
salivary glands	6.88 ± 0.032
midgut	6.68 ± 0.034
starved salivary glands	6.88 ± 0.058
starved midgut	6.72 ± 0.028
midgut luminal contents	5.97 ± 0.094

2.4 CONCLUSION

The salivary glands of the green mirid are large, implying they are important in digestive processes, and the anterior and posterior lobes of the principal salivary gland probably perform different functions. The midgut of the green mirid is a simple tubular structure. It is quite permeable, allowing basic and acidic dyes of at least 0.733 kDa and proteins of at least 44 kDa to pass from the lumen to the haemocoel. The slightly acidic environment of the midgut may be regulated by the ions stored in the concretions of the anterior midgut. The cells of the anterior and posterior midgut regions are structurally similar, although at an ultrastructural level the anterior cells contain more storage and possible secretory vesicles. The presence of these vesicles, plus the concentrations of mitochondria, suggest the anterior midgut of the green mirid plays an active role in the process of digestion. Functions of the two regions of the midgut of *C. dilutus* are still difficult to ascribe until the components of the vesicles are known.

CHAPTER 3: PROTEINASE ACTIVITY IN THE DIGESTIVE SYSTEM OF THE GREEN MIRID, *CREONTIADES DILUTUS*

3.1 INTRODUCTION

Mirids feed preferentially on the growing points of plants, using a lacerate-and-flush mechanism in which the stylets and watery saliva act to lacerate and flush out pockets of cells (Miles, 1972). Secreted saliva is believed to contain a variety of digestive enzymes that contribute to both penetration of the plant and to pre-oral digestion of the plant cells. In the mirid *Lygus disponsi* salivary gland digestive enzymes include an acidic proteinase (Laurema *et al.*, 1985). However, the most frequently reported proteolytic activity in the salivary glands of heteropteran insects is attributed to alkaline proteinases (Goodchild, 1952; Rastogi, 1962; Hori, 1970; Laurema *et al.*, 1985; Cohen, 1990). In the predatory bug *Zelus renardii,* the salivary gland proteinase activity has been characterised as a trypsin-like enzyme involved in pre-oral digestion (Cohen, 1993).

The extent of pre-oral digestion in phytophagous Heteroptera is unclear. While pectinases are commonly produced by the salivary glands of phytophagous heteropterans (Laurema *et al.*, 1985; Hori and Miles, 1993; Cohen and Wheeler, 1998), proteinase and amylase activity is apparently sometime present (Laurema *et al.*, 1985) and sometimes not (Cohen and Wheeler, 1998). It is difficult to draw conclusions since only a very few studies have looked for these enzymes in phytophagous Heteroptera. Most of the work that has examined digestive proteinases in heteropteran insects has focussed on haematophagous or predatory species.

In contrast with the salivary proteinases, the most common digestive proteinases in the guts of heteropteran insects have been suggested to be acidic proteinases of both the cysteine and aspartic classes (Terra and Ferreira, 1994). Cathepsin B and cathepsin D activities have been identified in the guts of several heteropteran families (Houseman, 1978; Houseman and Downe, 1982b; Houseman and Downe, 1983). However, alkaline proteinase activity has also been detected in the guts of several

Heteroptera (Goodchild, 1952, Hori, 1970, Takanona and Hori, 1974, Cohen, 1993). It is possible that these serine proteinases originated in the salivary glands, were secreted in the saliva and then ingested with the food. It remains unclear whether both cysteine and serine proteinases are commonly produced by cells in the guts of the Heteroptera.

The purpose of the work presented in this Chapter was to examine the enzymes present in the digestive system of the green mirid, a predominantly phytophagous member of the Heteroptera, as a measure of the level of pre-oral digestion, and to begin characterisation of any proteinases present particularly in relation to the evolution of these insects.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Phadebas tablets were purchased from Pharmacia Biotech (Uppsala, Sweden). Proteinase substrates azocasein, hemoglobin, *N*-benzoyl-L-tyrosine *p*-nitroanalide (BTpNA), *Nα*–benzoyl-DL-arginine *p*-nitroanilide (BApNA), *N*-succinyl-ala-ala-ala *p*-nitroanilide (SA_3pNA) and *N*-succinyl-ala-ala-pro-leu *p-nitroanilide* (SA_2PLpNA), were purchased from Sigma Chemical Co. (St Lois, Missouri). Proteinase inhibitors E-64, Antipain-dihydrochloride, Aprotinin, and AEBSF (4-(2-Aminoethyl)-benzenesulfonylfluoride, hydrochloride; Pefabloc® SC) were bought from Boehringer Mannheim GmbH (Germany). Standard molecular weight markers for electrophoresis, Coomassie® Brilliant Blue R-250, and the Bio-Rad Protein Assay concentrate, were purchased from Bio-Rad Laboratories (Hercules, California).

Absorbances were read on a Bio-Rad model 3550-UV Microplate Reader, or an Ultraspec II, model 4050 (LKB Biochrom Ltd, England).

3.2.2 Sample Preparation

Insects were collected and stored as described in Section 2.2.1. Adult mirids and third instar nymphs were removed at random from the laboratory population, and dissections were performed as described in section 2.2.2. The mirids were either dissected immediately for assaying, or, when comparing fed and starved mirids, placed in mesh covered boxes for 16 - 20 hours and given water, round beans and lettuce (fed mirids), or water only (starved mirids).

Salivary gland and midgut samples were prepared by dissecting the tissues from at least three mirids of the same developmental stage and feeding treatment (fed or starved) under cold insect saline solution (0.1 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $NaHCO_3$, pH 7). When required, tissues from male and female mirids were kept separate. The salivary glands were then combined in a microfuge tube with 50 µl of buffer appropriate to the assay and homogenised with a plastic pestle. The midguts were also combined in a microfuge and homogenised in 50 µl buffer. Homogenised

tissues were centrifuged for 5 minutes (min) at 8000 *g* at 4°C, and the supernatants were recovered.

Midgut contents were collected by removing the midgut from at least three adult mirids into cold insect saline solution. The contents were flushed out with gentle teasing and collected by a pipette into a microfuge tube.

Saliva was collected by allowing 30 adult mirids to feed on 15% sucrose sandwiched between a sheet of Parafilm® (American National Can™, Chicago Il) and a glass coverslip. After 16 - 20 hours, the coverslip was removed and the viscous liquid collected with a capillary tube. The resulting viscous mix of saliva and sucrose is referred to as saliva.

3.2.3 Protein assays

The concentration of protein in mirid extracts was measured according to the method of Bradford (1976), using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California) adapted to 96 well plates. Bovine serum albumin or bovine gamma globulin solutions of 0 - 25 µg/ml were used as standards.

3.2.4 α-Amylase assays

The method used to assay α-amylase activity was a modified version of an assay method provided by Dr. Peter Chandler (Division of Plant Industry, CSIRO). Five Phadebas tablets were ground to a powder with a mortar and pestle. Since this powder is not water soluble, separate tubes were set up for each time point of each sample. For salivary gland and gut extracts, four tubes were set up allowing a zero reading and three time points. In the case of saliva, limited sample meant that only two tubes, a zero reading and a single time point, were set up for each replicate. Into each microfuge tube was placed 20 mg of the Phadebas powder, 50 µl of Phadebas assay buffer (100 mM Na-succinate, 20 mM $CaCl_2$, pH 6.5) and 400 µl of sterile water. Finally, 50 µl of each sample, prepared at a concentration equivalent to 4 mirids per ml of distilled

water[†], was added. To one tube per sample, 1 ml of 0.1 M NaOH was immediately added to stop the reaction. The remaining assays were incubated at 37°C, and at set times one tube of each sample was removed and 1 ml of 0.1 M NaOH was added to it. Samples from each time point were mixed by inversion and centrifuged for 5 min at 720 *g* at room temperature. The absorbance of the supernatants was measured at 620 nm on a spectrophotometer to indicate the amount of dye released from the starch by the action of α-amylases. Six replicates of each sample were assayed.

3.2.5 General Proteinase Assays; Azocasein

Azocasein solution was prepared as described by Beynon (1989), and eqilibrated at 25°C for at least 15 minutes prior to use. Tissue samples and midgut contents were prepared at a concentration equivalent to 5 mirids per 166 µl of 0.15 M Tris-HCl (pH 8.0). To begin the reaction, 100 µl of sample (3 mirid equivalents) was added to 1 ml of azocasein solution. Immediately, 250 µl was removed from the reaction into 1 ml of cold 5% trichloroacetic acid (TCA) as the zero time blank. The remainder of each reaction was placed at 25°C for up to 24 hours. At three time intervals, which varied depending on the level of proteinase activity observed, a further 250 µl was removed into 1 ml of cold 5% TCA. Samples from each time point were centrifuged at 8000 *g* for 5 minutes at 4°C, and the absorbance of the supernatant was measured at 340 nm on a spectrophotometer. Control reactions using buffer in place of mirid extracts were included with each assay. Positive controls of trypsin were also included with most assays.

The effect of dithiothreitol (DTT) and ethylenediamine tetraacetic acid (EDTA) on proteinase activity was examined using the azocasein assay as described above. For this experiment, salivary glands and midguts were prepared at a concentration equivalent to 9 mirids per 320 µl of 0.15 M Tris-HCl (pH 8.0). 100 µl from each sample was then assayed with normal azocasein solution, 100 µl was assayed in the

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[†] A concentration equivalent to one mirid per ml is defined as the tissue, either the entire salivary gland complex, or midgut, or midgut luminal contents, from one mirid in one ml liquid.

presence of 3 mM DTT and 1 mM EDTA, and 100 µl was assayed in the presence of 5 mM DTT and 3 mM EDTA.

The effects of specific proteinase inhibitors (E-64, antipain, aprotinin, and AEBSF) were also tested using azocasein as follows. Two samples were combined to give the equivalent of 10 mirids per 333 µl. After assaying for protein concentration, a 100 µl aliquot was added to water only, and a second 100 µl aliquot was added to the inhibitor at an appropriate concentration (see Table 3.3). The samples were then incubated at 37°C for one hour before being added to the azocasein solution and assayed as described above.

3.2.6 General Proteinase Assays; Hemoglobin

An alternative assay with hemoglobin as substrate was used to examine the pH profile of mirid digestive proteinases, as hemoglobin is soluble over a wider range of pH conditions than azocasein. This assay was based on the method of Sarath *et al.* (1989), and used a hemoglobin solution prepared by dissolving 1 g of hemoglobin substrate powder in a solution of 16 g urea in 16 ml sterile water, and incubating at 37°C for one hour. To this was added 10 ml of 0.25 M Tris-HCl (pH 8), and the hemoglobin solution was divided into 8 aliquots of 3 ml each. The pH of each aliquot was adjusted (pH 2.0 to pH 11.0 in increments of 1.0) with either 1 M NaOH or concentrated HCl, then distilled water was added to a final volume of 4 ml. Hemoglobin solutions were equilibrated at 25°C for at least 15 minutes prior to starting the reaction.

Tissue samples and midgut contents were prepared at a concentration equivalent to 15 mirids per 75 µl of 0.25 M Tris-HCl (pH 8). The extracts were more concentrated than for azocasein-based assays due to the lower sensitivity of this assay. To begin the reaction, 50 µl of thermoeqilibrated hemoglobin solution was added to 10 µl of sample (2 mirid equivalents) and mixed gently. The reactions were incubated at 25°C for 16 - 20 hours, then stopped by the addition of 100 µl of 5% TCA. The stopped reactions were left on ice for 30 - 60 minutes, then centrifuged at 8000 *g* for 10

minutes at 4°C. The absorbance of the supernatant was measured at 280 nm on a spectrophotometer.

3.2.7 Specific Proteinase Assays

The trypsin and cathepsin B and H substrate BApNA, the elastase substrate SA_3pNA, and the chymotrypsin and elastase substrate SA_2PLpNA, were used to assay specific proteinases (Sandeman *et al.*, 1990). Tissue samples and midgut contents were prepared at a concentration equivalent to 3 mirids per 65 µl of 0.15 M Tris-HCl (pH 8). The midguts used in these assays were flushed to remove luminal contents before being homogenised. The reaction was started with the addition of 50 µl of sample to 50 µl of substrate (2 mM in 5% N,N'-dimethylformamide, (DMF)) in random order in a 96 well assay plate. Appropriate blanks were run as well as positive controls using trypsin or chymotrypsin. The plate was incubated at 30°C for up to 24 hours and the absorbance at 405 nm was monitored over time.

The chymotrypsin substrate BTpNA was also used to assay green mirid digestive tissues following the method described by Christeller *et al.* (1989). Salivary glands and intact midguts were prepared at a concentration equivalent to 6 mirids per 120 µl of 50 mM Tris-HCl, pH 8.0. Assays contained 100 µl of sample (5 mirid equivalents) and 1 mM BTpNA (in 20% DMF) in 1 ml of 50 mM Tris-HCl (pH 8.0). Immediately after starting the reaction, 500 µl was removed from the reaction into 230 µl of 30% acetic acid as the zero time blank. The remainder of each reaction was placed at 30°C for up to 20 hours, then stopped by the addition of 230 µl of 30% acetic acid. Samples from each time point were centrifuged at 8000 *g* for 5 minutes at 4°C, and the absorbance of the supernatant was measured at 410 nm on a spectrophotometer. Control reactions using buffer in place of mirid extracts were included with each assay.

3.2.8 Analysis of Assay Data

Standard curves, gradients and gradient means were calculated using StatView® SE+Graphics (Abacus Concepts, Inc., California). Analysis of Variance (ANOVA) and T-tests were used to compare the means of data from the azocasein assays using the

same software. ANOVA was also used to examine the effects of different samples on the hydrolysis of both starch and the specific proteinase substrates using Genstat 5, Release 3.1 (Sun 4 SPARC/ SunOS 5). For the α–amylase assays, specific activity data (absorbance per hour per mg protein) were transformed using natural logarithms to meet normality criterion prior to ANOVA testing.

3.2.9 Zymography

This method was kindly provided by Dr. R. Beynon (Department of Biomolecular Sciences, UMIST, UK). Tissue samples and midgut contents were prepared at a concentration equivalent to 3 mirids per 50 µl of distilled water. Samples were diluted with 2 x sample buffer (0.0625 M Tris-HCl, 10% Glycerol, 0.0125% Bromo-phenol Blue, 4% sodium dodecyl sulfate (SDS)) at either a 1:1 or 2:1 ratio (sample:buffer), then incubated at 37°C for one hour. Acrylamide gels (10% T; 3.33% C) were cast using the Mini-PROTEAN II ® system (Bio-Rad Laboratories), and were run according to the method of Laemmli (1970) with the following modifications. Acrylamide gels were copolymerised with 0.6% (w/v) gelatin. Following electrophoresis gels were soaked for 1 h in 2.5% TritonX-100 at room temperature, rinsed 3 times in distilled water, and incubated overnight at 37°C in refolding buffer (0.05 M Tris, 0.2 M NaCl, 0.05 M $CaCl_2$, 0.067% (w/v) Brij35, pH 7.6). Gels were rinsed again three times in distilled water and stained with Coomassie® Brilliant Blue R-250 (in 40% methanol, 10% glacial acetic acid, Bio-Rad Laboratories) for between 1 and 8 h. Finally, gels were destained for up to 24 h in 40% methanol, 10% glacial acetic acid, with clear zones against a blue background indicating proteinase activity towards the copolymerised gelatin.

Pre-stained molecular weight standards were included on each gel, and used to form a standard curve for the calculation of approximate molecular weights. These may not represent the actual molecular weights of the proteins because the zymograms were non-reducing.

3.3 RESULTS

3.3.1 α-Amylase assays

Salivary gland and midgut extracts from fed adult mirids, starved adult mirids, third instar nymphs, as well as secreted saliva, were assayed for the presence of α-amylase activity using Phadebas tablets as the substrate. The raw data for all α-amylase assays and their analyses are given in Appendix 2. Due to limited samples, only a single absorbance, at 3 hours, was measured for each of the six saliva replicates. This was used to calculate the mean α-amylase activity as change in absorbance per hour per mirid equivalent (Table 3.1), but it is noted that this activity may not have been linear over time.

For all other samples, the absorbance at 620 nm did increase linearly over time, indicating the presence of α-amylase activity. Both total activity (change in absorbance per hour per mirid equivalent) and specific activity (change in absorbance per hour per mg of extract protein) were determined (Table 3.1), and were higher in the salivary glands than in the midgut ($F_{1,20}=5$; $p<0.04$). No differences were found between adult mirids and third instar nymphs in either the total activity ($F_{1,15}=0.67$; $p=0.43$) or the specific activity ($F_{1,15}=1.07$; $p=0.32$) (Fig. 3.1 A). Also, no differences were found between fed and starved mirids in either the total activity ($F_{1,15}=0.04$; $p=0.84$) or the specific activity ($F_{1,15}=1.30$; $p=0.27$), although in general activity was higher after starvation (Fig. 3.1 B).

Table 3.1: Activity of green mirid digestive α-amylases against Phadebas powder.

	Total Activity (ΔOD per hour per mirid equivalent)	**Specific Activity** (ΔOD per hour per mg protein)
Saliva	0.0023 ± 0.00059	ND
Salivary glands	0.94 ± 0.18	35.5 ± 5.8
Midgut	0.50 ± 0.073	16.7 ± 4.0
Amylase†	0.24	47,900

Figures given are mean ± standard error (SE) of at least 5 determinations
† α-amylase positive control at 196 units/ml. Activities are per amount of α-amylase.
ND = not determined for the sample

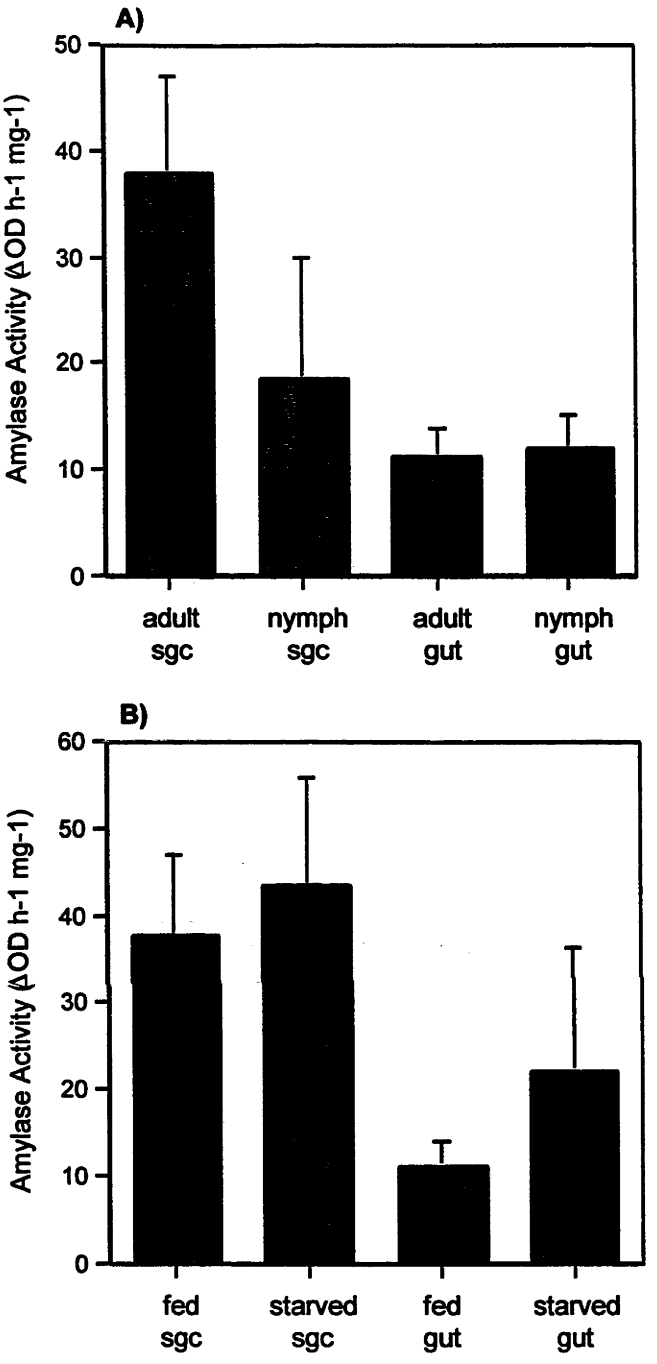

Figure 3.1: Measured α-amylase activity in salivary gland (sgc) and midgut (gut) extracts from the green mirid. Data shown are the mean specific activities (change in absorbance per hour per mg protein) ± SE of 6 determinations. Comparisons shown are between **A)** adult mirids and third instar nymphs, and **B)** fed and starved mirids.

3.3.2 General proteinase assays; Azocasein

Level of Proteinase activity:

General proteinase activity in salivary glands and midguts of adult mirids and third instar nymphs, and in the secreted saliva and midgut contents of adult mirids, was measured with azocasein as the substrate. The raw data and analyses for these assays are given in Appendix 3. Both total activity (change in absorbance per hour per mirid equivalent) and specific activity (change in absorbance per hour per mg of extract protein) were determined (Table 3.2). Low levels of proteinase activity were detected in all samples tested. Total activity was higher in both the salivary glands and saliva than in either the midgut or midgut contents ($F_{3,68}$=10.9; $p<0.001$). The specific activity was higher in the saliva and salivary glands than in the midgut, and higher in the saliva than in the salivary glands ($F_{2,54}$=69.4; $p<0.001$). Absorbance increased linearly over time with salivary gland extracts, but increased exponentially for all five secreted saliva samples examined. A single assay demonstrating this exponential increase is shown in Fig. 3.2. For the midgut extracts and midgut contents, the increase in absorbance over time was linear, although in 20% of samples there was some curvature, varying from very minor to an almost exponential increase.

Table 3.2: Activity of green mirid digestive proteinases against azocasein.

	Total Activity (ΔOD per hour per mirid equivalent)	**Specific Activity** (ΔOD per hour per mg protein)
Saliva	0.0053 ± 0.0014	2.061 ± 0.98*
Salivary glands	0.0059 ± 0.00053	0.339 ± 0.039
Midgut	0.0020 ± 0.00058	0.0297 ± 0.0069
Gut contents	0.0016 ± 0.00055	ND
Trypsin†	0.052 ± 0.0055	51.8 ± 5.5

Figures given are mean ± SE of at least 5 determinations

* Mean of only 2 determinations

† Positive control of 1 µg trypsin. Activities are per amount of trypsin.

ND = not determined for the sample

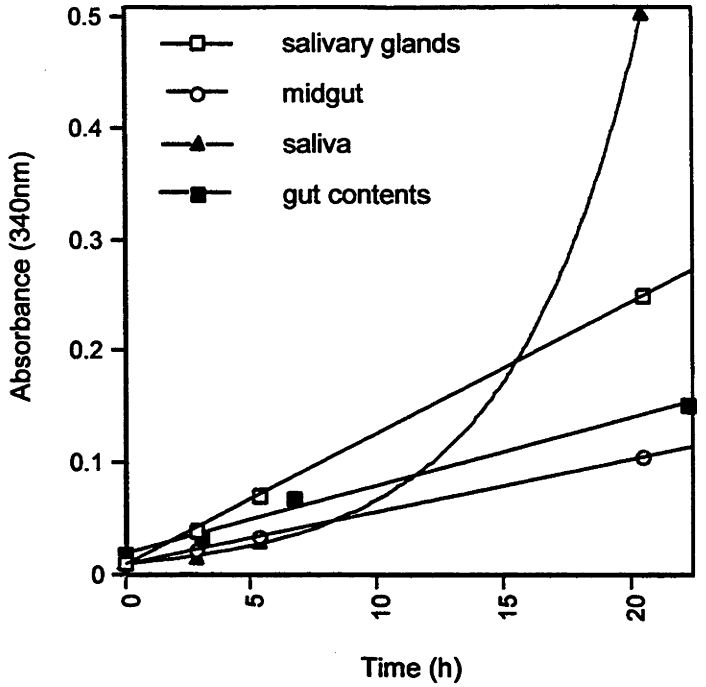

Figure 3.2: Activity of green mirid digestive proteinases against azocasein. Data is from a single assay, and linear or exponential lines of best fit have been drawn (salivary glands, $y=0.012x+0.008$, $r^2=1.000$; midgut, $y=0.005x+0.009$, $r^2=1.000$; saliva, $y=0.010*10^{0.084x}$, $r^2=0.998$; gut contents, $y=0.006x+0.019$, $r^2=0.991$)

Effect of DTT and EDTA:

The presence of DTT and EDTA caused a slight reduction in total proteinase activity in the salivary glands and midgut of the green mirid (Fig. 3.3). The differences observed however were not significant ($F_{2,20}=1.667$; $p=0.214$), and DTT and EDTA were omitted from further assays.

Proteinase Activity in Adult Mirids and Third Instar Nymphs:

Both total and specific proteinase activities in salivary gland and midgut extracts were compared between adult mirids and third instar nymphs (Fig. 3.4 A). The total proteinase activity was slightly higher in the salivary glands of nymphs than in those of adult mirids ($t_{31}=2.08$; $p=0.046$). There was no difference however in total proteinase in the midguts of third instar nymphs and adults ($t_{30}=0.562$; $p=0.58$). Also there were no differences between adults and nymphs in specific activity in either the salivary glands or midguts ($t_{28}<1.6$; $p>0.13$), and for all other azocasein assays adult mirids were used.

Proteinase Activity in Male and Female mirids:

Total and specific proteinase activities in salivary gland and midgut extracts were also compared between male and female mirids (Fig. 3.4 B), and no significant differences were detected ($t_{16}<1.5$; $p>0.17$). Interestingly, total activity was generally higher in females, while specific activity was slightly higher in males. The two sexes were pooled randomly for all other azocasein assays.

Proteinase Activity in Fed and Starved mirids

Proteinase activities were also compared between fed and starved mirids (Fig. 3.4 C), and no significant differences were found ($t_{>10}<1.4$; $p>0.21$). In general though, the salivary glands of fed mirids had a higher level of proteinase activity than those of starved mirids. The opposite was true for the midguts, with the starved mirids having more proteinase activity than the fed mirids.

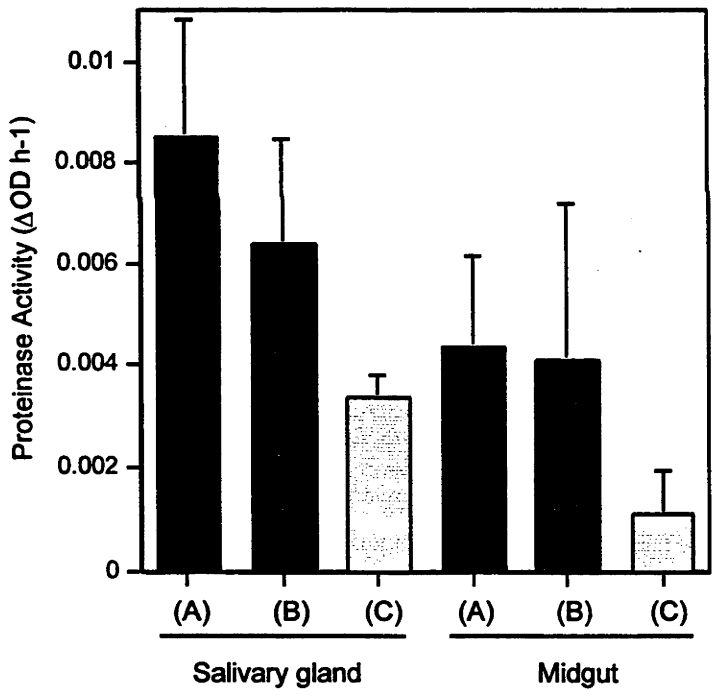

Figure 3.3: Effect of EDTA and DTT on total proteinase activity (change in absorbance per hour) in salivary gland and midgut extracts of the green mirid ± SE, as measured by azocasein hydrolysis. Treatments; (A) Tris-HCl buffer only, (B) 3 mM EDTA + 1 mM DTT, (C) 5 mM EDTA + 3 mM DTT

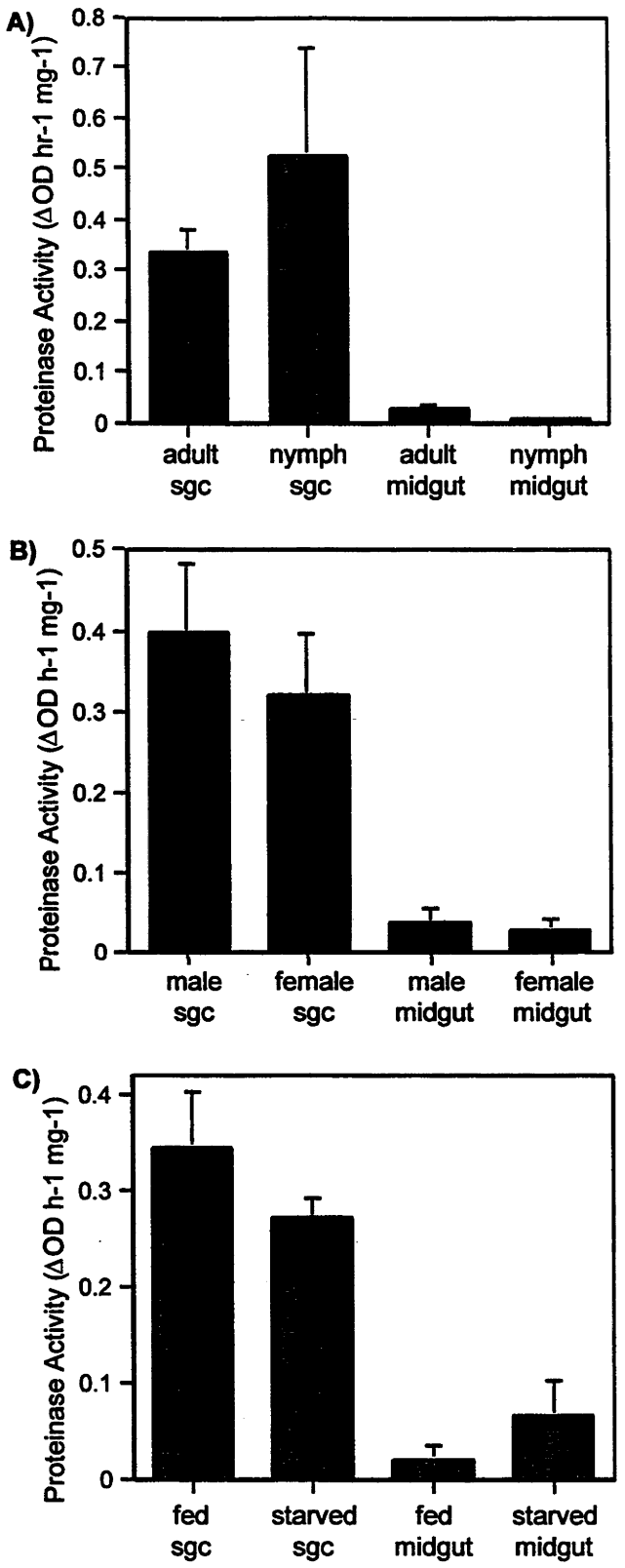

Figure 3.4: Activity of salivary gland (sgc) and midgut (gut) extracts from the green mirid against azocasein. Data shown is the mean specific activity ± SE of at least 4 determinations. Comparisons are between **A)** adult mirids and third instar nymphs, **B)** male and female mirids, and **C)** fed and starved mirids.

Effects of Proteinase Inhibitors:

The effects of specific inhibitors on the activity of the digestive proteinases of the green mirid are summarised in Table 3.3. In both the salivary glands and midgut the serine proteinase inhibitor aprotinin almost completely inhibited proteinase activity. Antipain, which inhibits trypsin, papain and cathepsins A and B, reduced proteinase activity by about 40% in both tissues. The cysteine proteinase inhibitor E-64 showed slightly more variation, reducing midgut proteinase activity by 48%, salivary gland proteinase activity by 33%, and saliva proteinases by 60%. AEBSF, a serine proteinase inhibitor, showed the largest variation in effect on proteinase activity in the different samples. AEBSF caused only a 21% reduction in midgut proteinase activity, but inhibited salivary gland proteinases by 56% and saliva proteinases almost completely.

Table 3.3: The effect of inhibitors on hydrolysis of azocasein by extracts from the digestive system of the green mirid.

Inhibitor	Conc.*	Salivary Glands†	Midgut†	Saliva†
None		100	100	100
AEBSF	0.25	43.67 ±1.5	78.59 ± 20.5	0.50 ± 0.5‡
Aprotinin	0.002	3.23 ± 1.7	0	ND
Antipain	0.05	56.66 ± 6.7	58.27 ± 14.1	ND
E-64	0.05	66.66 ± 11.3	52.22 ± 10.0	39.58 ± 13.7‡

* Concentration of the inhibitor in mg/ml

† Figures given are mean percentage activity remaining relative to unihibited controls ± SE of at least 3 determinations.

‡ Mean of only 2 determinations

ND = not determined for the sample

3.3.3 General proteinase assays; Hemoglobin

The activity of proteinases in the saliva, salivary glands and midguts of mirids was assayed over a range of pH values from pH 2.0 to pH 11.0 using hemoglobin as substrate (Fig. 3.5). The raw data for the hemoglobin assays is given in Appendix 4. The salivary gland proteinases had a broad optimum from pH 3.0 to 10.0, peaking

slightly at pH 8.0. In contrast, the midgut proteinases had a sharp optimum at pH 3.0 to 4.0, with considerably less activity at the higher pH values. Saliva had highest proteinase activity at pH 10.0.

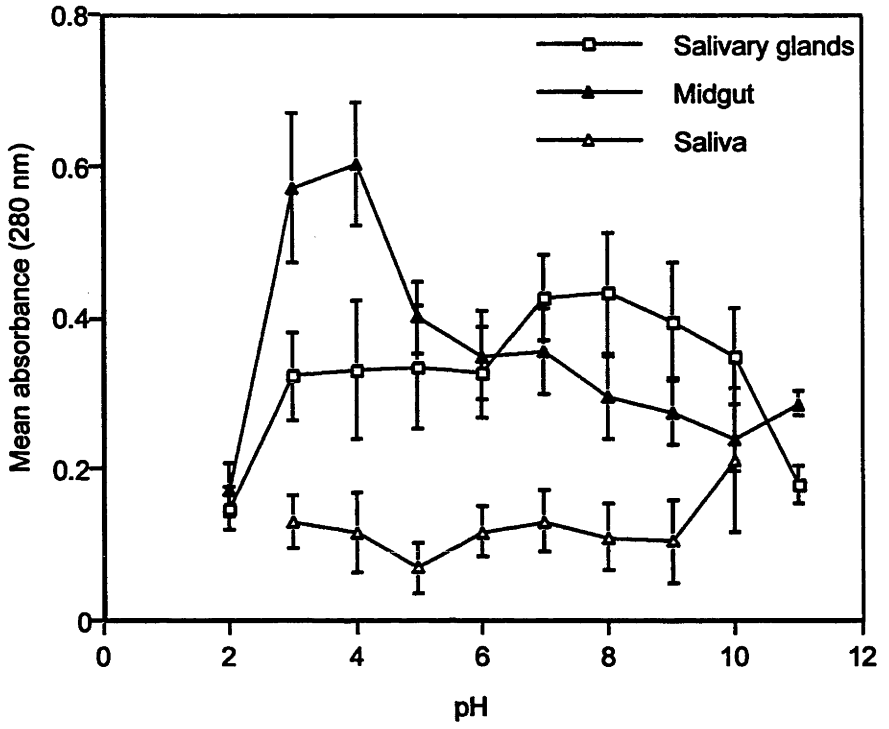

Figure 3.5: pH profile of green mirid proteinases. Activity of green mirid salivary glands, midgut and secreted saliva against hemoglobin over a range of pH conditions. Points represent the mean absorbance ± SE of at least 3 determinations.

3.3.4 Specific Proteinase Assays

Mirid digestive proteinases were assayed with the substrates BApNA, BTpNA, SA_3pNA and SA_2PLpNA (Table 3.4). ANOVA was used to examine the effects of different samples on the hydrolysis of these substrates. The raw data of all specific proteinase assays and their analyses are given in Appendix 5. For the whole mirid, hydrolysis of SA_2PLpNA was higher than for any other specific substrate tested ($F_{2,55}=27$; $p<0.001$). Overall activity against pNA substrates was highest in the salivary glands ($F_{1,55}=36$; $p<0.001$). However there was also a significant interaction effect ($F_{5,55}=32$; $p<0.001$), meaning different substrates were hydrolysed to varying extents in the different tissues.

Hydrolysis of BApNA was higher in the midgut contents than in either the salivary glands or midgut tissue. Against all other specific substrates tested, the salivary glands had higher activity than the midgut or midgut contents (where tested). Salivary activity was lowest against SA_3pNA, implying only low levels of elastase-like activity in the digestive system of the green mirid. Hydrolysis of BTpNA by salivary glands suggests the presence of chymotrypsin-like activity, which is probably contributing to the high levels of hydrolysis against SA_2PLNA. Secreted saliva also showed very high levels of hydrolysis of both SA_3pNA and SA_2PLpNA, suggesting the presence of elastase-like, and possibly chymotrypsin-like, activities. However, the total activity of saliva against both SA_3pNA and SA_2PLpNA (0.0012 and 0.0029 nmol pNA per min respectively) was a lot lower than the specific activity due to the low amounts of protein in the sample.

Table 3.4: Activity of green mirid digestive proteinases against synthetic substrates.

	BApNA	BTpNA	SA_3pNA	SA_2PLpNA
Saliva	0*	ND	17.1*	8.86*
Salivary glands	0.12 ± 0.043	0.39 ± 0.057	0.10 ± 0.025	5.21 ± 0.82
Midgut	0.17 ± 0.046	0.00056 ± 0.0026	0.013 ± 0.0028	0.33 ± 0.089
Gut contents	2.21 ± 0.61	ND	0.055 ± 0.0089	0.24 ± 0.088

Figures given are mean nmoles of *p*-nitroanalide released per min per mg protein ± SE of six determinations

* Figure given is of a single determination

ND = not determined

3.3.5 Zymography

Zymograms were used to investigate the number and relative sizes of the proteinases present in the digestive tissues of the green mirid. Note that the molecular weights (MW) calculated may not represent the actual MW of the protein, and they are given here as a means of comparing activities between samples only. A total of 32 salivary gland extracts (three mirids each), 25 midgut extracts (three mirids each), and

6 saliva samples (each collected from 30 mirids) were electrophoresed on zymograms (Table 3.5). The results varied within tissues, as well as among different tissues and saliva, and a typical result is shown in Fig. 3.6. Across all salivary gland samples, eight bands of different MW were observed. Six bands could occasionally be found in a single extract, but it was more usual to see between two and four bands of activity per extract. Two bands with approximate MW of 69.4 and 30.2 kDa were present in all salivary gland extracts examined. Two other bands, at 53.2 and 44.4 kDa, were seen in eleven and twelve of the salivary glands extracts respectively.

Eight different sized bands were observed in midgut extracts. Usually only one or two bands were present per extract, and in some cases no active bands could be seen. No single band was present in all midgut extracts, with the most common band (MW 53.2 kDa) being seen in 14 of the 25 extracts. A band of 30.2 kDa was present in ten of the extracts. In the secreted saliva two strong bands were commonly observed. One band had a MW of 52.6 kDa and was present in all six samples, and the second band, MW 59.0, was present in four of the samples.

Table 3.5: Summary of proteinase activities detected by zymography.

Sample	Approximate molecular weight (kDa)	Number of times observed
Salivary glands	142.1	2
(32 extracts in total)	109.3	2
	69.4	32
	57.5	4
	53.2	11
	44.4	12
	30.2	32
	16.9	5
Midgut	142.1	3
(25 extracts in total)	107.9	2
	88.1	2
	69.9	5
	62.1	3
	53.2	14
	30.2	10
	14.6	4
Secreted saliva	59.0	4
(6 samples in total)	52.6	6

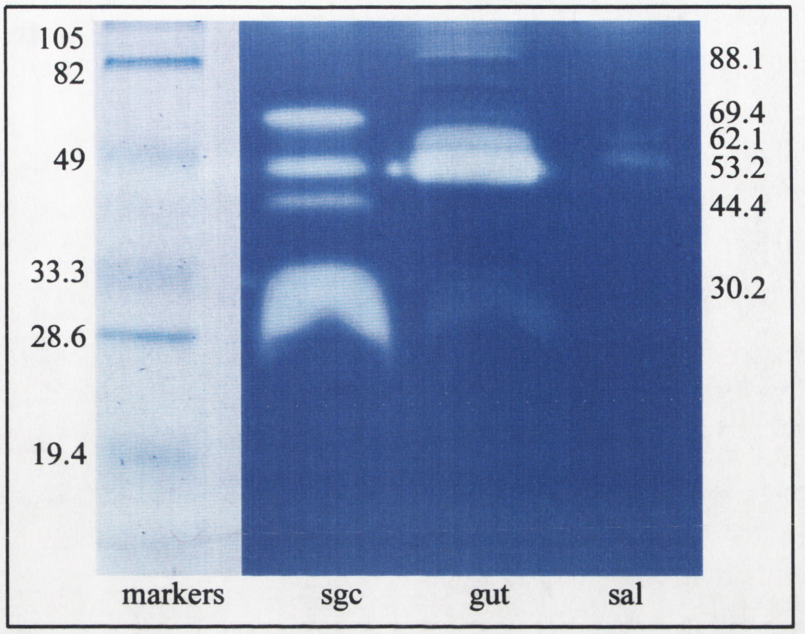

Figure 3.6: Zymogram of mirid digestive proteinases, showing prestained molecular weight Markers, salivary gland extracts (SGC), midgut extracts (Gut), and secreted Saliva. Approximate molecular weights in kDa are shown on the left for the marker proteins, and on the right for the major proteinases detected.

To further localise the proteinase activity in the mirid digestive system, the salivary glands were separated into the accessory gland, the anterior lobe of the principal gland, and the posterior lobe of the principal gland. The midgut was separated into two sections, anterior and posterior, and as much as possible of the contents of the lumen were removed and kept separate. These samples were then run in individual lanes on zymograms. In the salivary glands, the two major proteinase bands were present in all sections of the salivary gland complex, but were generally most abundant in the posterior lobe of the principle gland (Fig. 3.7). This observation was consistent over the 3 zymograms examined. In the midgut samples, the lumen contents consistently had most of the proteinase activity (Fig. 3.8).

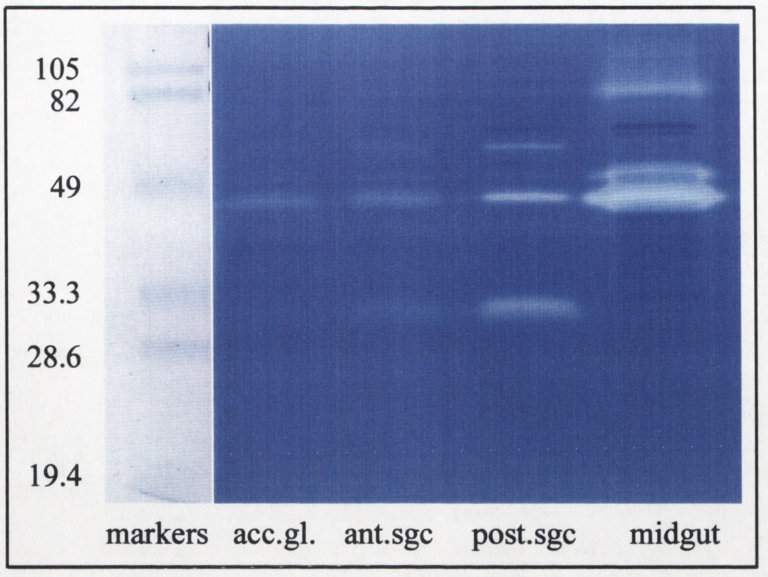

Figure 3.7: Zymogram showing extracts from the green mirid accessory salivary gland (acc.gl.), anterior lobe of the principal salivary gland (ant.sgc), posterior lobe of the principal salivary gland (post.sgc), and entire midgut (midgut). Molecular weights of the marker proteins are given on the left in kDa.

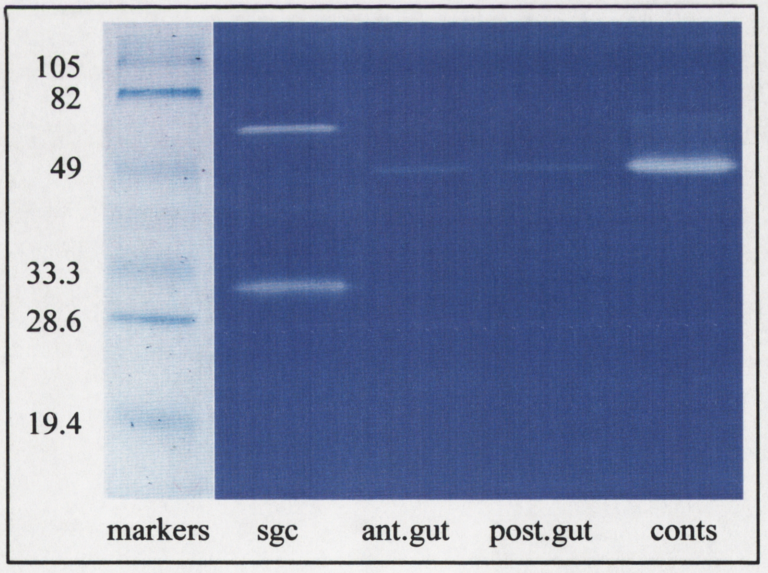

Figure 3.8: Zymogram showing extracts from the green mirid salivary gland complex (sgc), anterior flushed midgut (ant.gut), posterior flushed midgut (post.gut), and midgut luminal contents (conts). Molecular weights of the marker proteins are given on the left in kDa.

3.4 DISCUSSION

Most studies examining the digestive system of heteropteran insects have focussed on hematophagous or predatory species. One exception is the study by Laurema *et al.* (1985) of the salivary enzymes of the plant-feeding mirid *Lygus disponsi*. In this study it was observed that the three most important secretable salivary gland enzymes were polygalacturonase (pectinase), amylase, and alkaline proteinase. Pectinase activity has previously been demonstrated in the saliva of the green mirid (Hori and Miles, 1993), and part of the aim of this Chapter was to examine whether α-amylase and proteinase are active, and if so in which tissues.

The salivary glands, midgut and secreted saliva from the green mirid all demonstrated α-amylase activity. The salivary glands had higher α-amylase activity than the midgut, implying that salivary α-amylases are important in the digestive processes of the green mirid. In fact, it is possible that the α-amylase activity detected in the midgut is due to an α-amylase which originated in the salivary glands and was ingested with the food, remaining active in the gut.

Proteinase activity, as indicated by azocasein digestion, was detected in the midgut, midgut contents, salivary glands and saliva of the green mirid. This assay was performed at pH 8.0, and so may favour the detection of alkaline proteinases, although some cysteine proteinases also retain activity in these conditions (Liu *et al.*, 1997). The measured activity was quite low, being at least 200-fold lower in the midgut of the mirid than in the gut of the lepidopteran *Heliothis virescens* (Johnston *et al.*, 1995). It is not surprising that the proteinase levels in the green mirid are low. Of the few attempts to identify proteinase activity in phytophagous Heteroptera, several have been unsuccessful (Goodchild, 1952; Nuorteva, 1954; Cohen and Wheeler, 1998) implying the absence, or at least extremely low levels, of proteinase activity in these insects. Proteinase activity was also difficult to detect in the green mirid by standard azocasein assay methods, and it was necessary to substantially increase sample concentrations and incubation times to obtain reliable measurements of proteinase activity.

The salivary glands and saliva of the green mirid had higher levels of proteinase activity than either the midgut or midgut contents, implying that the salivary gland

complex is a major source of digestive proteinases in the green mirid. This in turn suggests that pre-oral digestion mediated by salivary gland enzymes may be important for the plant feeding mirid *C. dilutus* as well as for predatory bugs such as the reduviid *Z. renardii* (Cohen, 1993).

The exponential increase in proteinase activity over time observed in the saliva could be explained by the presence of an inactive form of the proteinase, a zymogen, which becomes activated over time (Moffatt and Lehane, 1990). The exponential increase in proteolytic activity which was occasionally observed in the midgut extracts and midgut contents could have resulted from the activation of a zymogen present in the saliva which passed into the midgut with the food. The use of salivary proteinases to continue digestion in the gut has been suggested previously for heteropteran insects (Houseman and Downe, 1983; Cohen, 1993). However, the data presented does not exclude the possibility of either the same or a different zymogen being present in some samples which originated from the gut cells.

Thiol compounds such as DTT can protect cysteine proteinases from inactivation, and hence enhance their activity (Barrett, 1977). Also, EDTA can act to stabilise some cysteine proteinases (Applebaum *et al.*, 1964), so the combined effect of DTT and EDTA on mirid proteinases was examined. Together at reasonably high concentrations these compounds actually caused a slight reduction in mirid proteinase activity, possibly due to the inhibition of some metallo-proteinases by EDTA.

Nuorteva (1954) was unable to detect proteinase activity in the salivary secretions of *Lygus rugulipennis* adults, but could do so in nymphs. Conversely, in *L. disponsi,* salivary proteinase activity in third instar nymphs was higher than in adults, and in general varied throughout development (Hori, 1970). The disparity in the results of the different studies may be due to variation in proteinase levels between insects or even within insects, or to species variation. In addition, the use of different assay techniques makes comparisons between studies difficult. In the green mirid, total proteinase activity was higher in the salivary glands of third instar nymphs than in adults, in agreement with the study of *L. disponsi.* The specific activity did not vary

however, and so the difference observed may be due to the fact that the salivary glands of the nymphs were often larger than those of the adults.

There were no differences detected in proteinase activity between male and female mirids, which is also the case in *L. disponsi* (Hori, 1970). In *L. rugulipennis* however, total proteinase activity is higher in the salivary glands of female insects than in males (Varis *et al.*, 1983). This does agree with the general trend in the green mirid where total proteinase activity is higher in females than in males, although the differences are not significant. The higher level of total proteinase activity in females compared to the higher level of specific activity in males is presumably due to the larger size of the females resulting in the presence of more protein in each assay, and hence higher total activity and lower specific activity.

In *Z. renardii* (Heteroptera: Reduviidae) feeding results in a depletion of salivary gland proteinases and an increase in midgut trypsin-like activity (Cohen, 1993). This is opposite to the trend observed in the green mirid, where any depletion observed after feeding is in the midgut and not the salivary glands. The differences observed however are not statistically significant, which may mean that proteinase synthesis is constitutive rather than being induced by food or a secretogogue. Similarly, in *L. disponsi* no differences in proteinase activies were observed between fed and starved insects (Hori, 1970). Plant feeding insects such as *L. disponsi* or the green mirid, unlike predators which obtain food in bursts, can essentially feed at any time, and constitutive synthesis of enzymes may be advantagous. This explanation is not universal though, since in the plant feeding mirid *L. rugulipennis,* salivary proteinase activity was depleted by starvation (Varis *et al.*, 1983).

Inhibitors, pH optima and specific substrates were used to partially characterise the proteinases present in the digestive system of the green mirid. The pH optimum for the proteinase activity in the salivary glands was alkaline (pH 8). The pH of the watery saliva of the green mirid was not measured, but in other hemipteran insects has been found to be slightly alkaline (Miles, 1965). The salivary gland proteinases then would have maximal activity in the secreted saliva. It is interesting that the pH optimum of the proteinases detected in secreted saliva is more alkaline at pH 10 than that of the

salivary gland proteinases. One explanation is that the zymogen which becomes active in the saliva may not be active in the salivary glands, and may have a higher pH optimum than salivary gland proteinases. Alternatively, the intracellular housekeeping proteinases in the salivary gland cells may have a lower pH optimum than the secreted proteinases present in the saliva, and these may be masking the presence of the more alkaline proteinases. In either case, it appears that different proteinases predominate in the salivary glands compared with the secreted saliva, which is supported by the different banding patterns observed on zymograms.

The alkaline pH optima for the salivary glands and saliva suggests the predominance of serine proteinases, in agreement with previous studies of heteropteran insects (Laurema *et al.*, 1985; Cohen, 1993). The serine proteinase inhibitors AEBSF and aprotinin substantially reduced salivary proteinase activity, supporting the presence of serine proteinases in the salivary glands and secreted saliva. In addition, aprotinin is quite specific for trypsin-like enzymes. However, the high level of hydrolysis of BTpNA and SA_2PLpNA, compared to the lesser hydrolysis of SA_3pNA and BApNA, implies salivary gland proteinase activity is chymotrypsin-like rather than elastase- or trypsin-like. This discrepancy may be explained by synergy or co-dependance of different proteinases. For example, small amounts of trypsin may be required to activate the chymotrypsin which then predominates. This situation differs from that of *Z. renardii,* in which trypsin-like activity was common and chymotrypsin-like activity was low. The difference may be due to the different lifestyles of the insects, that is phytophagy versus predation, since they would encounter different compounds including proteinase inhibitors in their diets. The presence of different proteinase inhibitors in the diet can alter the complement of digestive proteinases in insects (Broadway, 1995; Jongsma *et al.*, 1995).

There was also substantial salivary gland proteinase activity in more acidic pH conditions, in which serine proteinases are not usually active. In addition, some inhibition of green mirid salivary proteinases by E-64 occurred, indicating the presence of cysteine proteinase activity. If so, the cysteine proteinase present may be a lysosomal proteinase not involved in digestion, as was suggested for the cathepsin D-

like enzyme detected in *L. rugulipennis* (Laurema *et al.*, 1985). Inhibition by E-64 however is not absolute proof of cysteine proteinase activity. Amarant *et al.* (1991) suggested that a free cysteine in the active site of thrombin-like serine proteinases (achelases) in the caterpillar *Lonomia achelous* was resulting in inhibition by thiol proteinase directed reagents including E-64. Lee and Anstee (1995) found that a trypsin-like enzyme from the guts of *Spodoptera littoralis* was inhibited by E-64, and suggested this could be due to sequence homology to achelases. Possibly, rather than an active cysteine proteinase being present in salivary glands of the green mirid, E-64 is cross inhibiting a serine proteinase.

High SA_3pNA hydrolysis by secreted saliva suggests the presence of active elastases also. Elastase activity has not been reported in the saliva or salivary glands of heteropteran insects, though it is not uncommon in insect guts (Christeller *et al.*, 1990). However, due to the low amount of protein in saliva compared to that of salivary gland or midgut tissues, the value calculated for amount of *p*-nitroanalide released per mg in the saliva is perhaps deceptively high. The total level, on a per mirid basis, was lower and more comparable with the low amount of elastase in the other tissues. This is true for the SA_2PLpNA hydrolysis also, which may be due to either chymotrypsin- or elastase-like enzymes.

In the midgut, the pH optimum of the proteinase activity was acidic (pH 4). The pH of the luminal contents of the midgut of the green mirid is slightly acidic (approximate pH 6). This is not optimal for the midgut proteinases, although they maintain approximately half their maximum activity under these condition. Interestingly, under these conditions, both midgut proteinases and salivary proteinases have similar levels of activity, therefore both could be active in the midgut of the mirid.

The acidic pH optimum for the midgut proteinases indicates cysteine or aspartic proteianses predominate. The effect of E-64 on midgut proteinases supports the presence of cysteine proteinase activity. The predominance of acidic proteinases is also consistent with the minor effect of AEBSF on midgut proteinase activity, and with the observation that acidic proteinases are common in the midguts of heteropteran insects (Terra and Ferreira, 1994). Surprisingly, aprotinin inhibited midgut proteinase activity

completely, and this compound is believed to be serine proteinase specific, suggesting both serine and cysteine proteinases are active in the gut, although the serine proteinases detected could be salivary proteinases which have been ingested rather than midgut originating enzymes. The reduction in activity caused by antipain could result from the presence of either trypsin-like or cysteine proteinases. The high level of activity against BApNA by midgut samples does not help identify the predominant class of proteinases, since hydrolysis of this substrate could be due to either trypsin-like or cathepsin-like enzymes.

Multiple activities were detected on zymograms of salivary gland and midgut extracts. This technique may only detect serine (not acidic) proteinases, because hydrolysis occurred in buffer of pH 7.6, so a limited number of zymograms were incubated in a refolding buffer of pH 3.5 and pH 5.0. Overall band intensity was reduced in these conditions, but no change in banding pattern was observed. In saliva, two bands are usually present. These could be secreted from the salivary glands as separate proteinases. Alternatively, just the larger proteinase could be secreted, then cleaved to form the smaller proteinase. The third possibility is that a precursor (not detected by the zymogram) could be secreted and modified to give the two active bands observed. While the bands observed in the saliva and salivary glands almost certainly represent proteinases produced by the cells of the salivary glands, it is not possible from this data to determine the origins of the proteinases detected in the midgut. For example, the major band observed in the gut comigrates with one from both the salivary glands and saliva, and could have originated in the salivary glands and then been ingested with the food.

In summary, the majority of proteinase activity in the digestive system of *Creontiades dilutus* is salivary chymotrypsin-like activity, suggesting that pre-oral digestion is a major component of protein digestion in *C. dilutus*. Gut proteinases showed a different pH profile and inhibitor and substrate specificities to salivary proteinases, and the general activity was lower and more difficult to characterise. Cysteine proteinases probably account for much of the activity in the midgut of *C. dilutus*, consistent with previous studies of heteropteran insects (Houseman, 1978;

Houseman and Downe, 1982b; Houseman and Downe, 1983; Silva and Terra, 1994). However, data presented here does not exclude the presence of serine proteinases in the gut of the green mirid. Inhibitors and substrates may not be specific or effective enough to class a proteinase definitively even in purified enzyme preparations (Barrett and Heath, 1972). Impure preparations, such as the tissue homogenates examined here, provide special problems. In this case, the possible presence of ingested salivary proteinases in the midgut complicates the issue even further. Another significant problem is the low level of proteinase activity and the low sensitivity of the azocasein assay. This highlights a problem that may be common to other studies. It could be that classes of proteinases are missed due to difficulty in detection, and then assumed to be absent. It is therefore important to look for the different classes of proteinases specifically, and with sensitive techniques. A molecular approach, looking at the genes encoding proteinases in different groups of Heteroptera, may provide more definitive answers, and lend support to the biochemical analyses.

CHAPTER 4
CLONING OF PROTEINASE GENES FROM THE GREEN MIRID, *CREONTIADES DILUTUS*.

4.1 INTRODUCTION

Detection and biochemical characterisation of proteinase activity is based largely on general proteinase activity assays, inhibitor and substrate specificities, pH optima, and sometimes purification of the proteinase of interest. The results presented in Chapter 3 highlight some of the problems associated with the application of these approaches, especially to small insects such as *Creontiades dilutus*. Some general assay techniques simply are not sensitive enough to detect low levels of activity. This is exacerbated by the fact that proteinase activity can vary not only between insects, but also at different times during the development of an individual insect (Hori, 1970), making it difficult to confirm the presence or absence of proteinase activity. Another difficulty with proteinase assays lies in determining the original location of any active proteinases detected. For example, the proteinase activity detected in the midgut of the green mirid could originate in the salivary glands and be ingested with the food, or it could originate in the midgut cells themselves.

Specific inhibitors and substrates are important tools for the characterisation of proteinases into mechanistic classes. However, the presence of multiple proteinases in a sample and the existence of "dual-specificity" proteinases (Macaldowie *et al.*, 1998) can cause confusion, plus there is the potential for cross inhibition as discussed in Chapter 3. pH optima of proteinase classes can overlap and so are also not definitive alone. Finally, purification of proteinases for sequence based classification or protein localisation can be difficult due to the possibility of autocatalysis.

To date no molecular work on digestive enzymes of the Heteroptera has been published. Molecular studies of insect digestive proteinases have mainly focussed on the serine proteinase genes of Diptera and Lepidoptera. Some studies of cathepsin L-like cysteine proteinase genes from insects also exist. The sequence data from these studies has allowed the development of a polymerase chain reaction (PCR)-based

strategy for gene identification in this Chapter. The aim of the studies reported in this Chapter is to examine the genes encoding digestive proteinases in the green mirid in terms of the classes represented and the location of expression, to complement the biochemical analysis.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Degenerate primers, Taq DNA polymerase, and RNA markers (0.24-9.5 kilobase (kb) ladder) were purchased from Gibco BRL. M13 forward and reverse primers, and specific primers for sequencing, were purchased from Bresatec. Restriction enzymes and buffers were purchased from New England Biolabs. Nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Promega. Proteinase K, digoxygenin (DIG)-labelled-UTP, and the anti-DIG-alkaline phosphatase conjugate, were purchased from Boehringer Mannheim. PBC KS+ phagemid vector was purchased from Stratagene.

4.2.2 DNA Manipulation

Standard manipulations of DNA, including ligation, restriction enzyme digestion, alkaline lysis mini-prep purification of plasmid DNA, phenol and chloroform extractions, ethanol precipitation, and agarose gel electrophoresis, were done as described in Sambrook *et al.* (1989). Electroporation of *E.coli* cells was done following the method of Ausubel *et al.* (1994).

4.2.3 Southern Blot Analysis

4.2.3.1 Transfer of DNA

For analysis of the inserts of multiple clones resulting from ligation of PCR product, digested plasmid DNA was electrophoresed on a 0.8% agarose gel, then transferred to GeneScreen Plus (NEN-DuPont) positively charged nylon membrane using the alkaline transfer method of Ausubel *et al.* (1994).

4.2.3.2 Hybridisation

Membranes were wet with 6 x Sodium Chloride/Sodium Citrate buffer (SSC) (1 x SSC; 0.15 M NaCl, 15 mM Na_3 Citrate.$2H_2O$, pH 7.0), and prehybridised in aqueous hybridisation solution (5 x SSC, 5 x Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 0.2 mg/ml denatured salmon sperm DNA) for at least one hour prior to addition

of 200 µl of probe. Hybridisations were performed at 65°C in Hybaid hybridisation ovens in Hybaid glass bottles. Membranes were washed 3 x 30 min in 0.5 x SSC, 0.1% (w/v) SDS at 65°C, sealed in plastic, and exposed to *new* RX (Fuji) X-ray film at -80°C.

4.2.4 Northern Blot Analysis

Approximately 1 µg of mRNA, prepared from total RNA (see 4.2.6), and 4.5 µg RNA markers, were electrophoresed on an 18% formaldehyde/1.4% agarose gel as described in Ausubel *et al.* (1994). RNA was blotted onto GeneScreen Plus (NEN-DuPont) positively charged nylon membrane overnight in 10 x SSC. The membrane was rinsed in 6 x SSC and baked for 2 hours at 80°C.

After baking, membranes were rinsed in 6 x SSC and prehybridised for 4 hours at 65°C in Nasmyth's hybridisation solution (1.1 M NaCl, 0.3 M Na_2HPO_4, 0.011 M Na_2EDTA, 1.85% N-Lauryl sarcosine, 18.5% dextran sulphate, pH 6.2), diluted 1/1.85 before use. 200 µl of denatured DNA probe (green mirid salivary gland serine proteinase gene, *CdSp1*) (see 4.2.5) was then added, and left to hybridise as described in section 4.2.3.2 for 16 hours. Membranes were washed 3 x 30 min in 0.5 x SSC, 0.1% (w/v) SDS at 65°C, sealed in plastic, and exposed to *new* RX (Fuji) X-ray film at -80°C.

4.2.5 Probe Synthesis

Hybridisation probes were prepared by radiolabelling DNA with [α^{32}P]-dATP by random primed synthesis using the NEBlot kit (New England Biolabs) as described by the manufacturer.

4.2.6 Isolation of mRNA from Total RNA

Total RNA was isolated from 0.88 g of whole mirids (approximately 110 mirids) using the Ultraspec™-II RNA Isolation System (Biotecx Laboratories Inc.). Mirids were homogenised in 5 ml of Ultraspec™ RNA reagent with a polytron homogenizer, and the RNA was extracted and purified as described by the

manufacturer. Half of the purified total RNA was diluted to 1 ml with Tris/EDTA (TE) buffer (10 mM Tris.Cl pH7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), pH8.0), and mRNA was purified from this total RNA using the mRNA Purification Kit (Pharmacia-Biotech) as described by the manufacturer. The purified mRNA was quantitated by spectrophotometry and used for Northern blot analysis and to make cDNA for library construction.

4.2.7 Isolation of mRNA for RT-PCR

mRNA was purified from the salivary gland complexes and midguts of 40 adult green mirids using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). Tissues were ground in microfuge tubes in 400 µl of Extraction Buffer initially, then mRNA was extracted according to the manufacturer's instructions. Purified mRNA was eluted in 200 µl of Elution Buffer and quantitated by spectrophotometry. The mRNA was recovered by ethanol precipitation and resuspended in diethylpyrocarbonate-treated water for use in a reverse transcription polymerase chain reaction (RT-PCR).

4.2.8 RT-PCR

4.2.8.1 PCR strategy

A PCR-based strategy, involving either nested or half-nested PCR, was used to identify proteinase genes from the green mirid. In nested PCR two sets of oppositely directed primers are used sequentially to amplify the region of DNA flanked by both sets of primers (Fig. 4.1 A). In half-nested PCR, two primers of the same direction are used sequentially with a single primer of the opposite direction (Fig. 4.1 B). Using more than one PCR reaction with different primer sets greatly increases the specificity of the PCR.

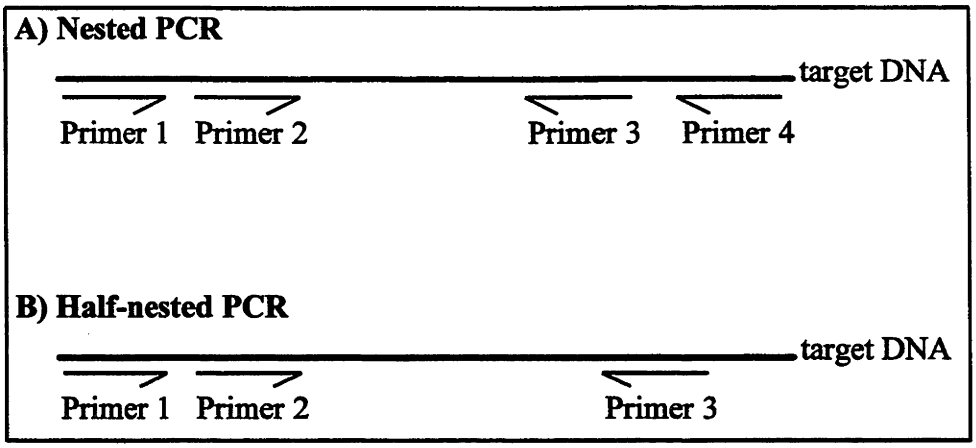

Figure 4.1: Nested and half-nested PCR strategies. **A)** In nested PCR the outer primer set, Primer 1 and Primer 4, are used in the first amplification reaction, and the inner primer set, Primer 2 and Primer 3, are used for a second amplification reaction. **B)** In half-nested PCR the outer primers, Primer 1 and Primer 3, are used in the initial reaction, and Primer 2 and Primer 3 are used in the second amplification reaction.

4.2.8.2 Cysteine Proteinase Primers

The amino acid sequences of the silk moth cysteine proteinase, *Drosophila melanogaster* putative digestive cysteine proteinase, fleshfly cathepsin L cysteine proteinase, maize weevil cysteine proteinase, and papain, were compared in a multiple sequence alignment using the PILEUP algorithm of the GCG software package (Genetics Computer Group, Program Manual for the Wisconsin Package, Version 8, August 1994, Madison, WI) (Fig 4.2). Conserved sequences were identified and used to design three degenerate primers that could be used in a half-nested PCR strategy. Primers Cys 1 and Cys 2 prime the template in the sense orientation. Primer Cys 3 primes in the antisense orientation, and in the *Drosophila* gene, forms a product 500 base pairs (bp) in length with Cys 1, and 374 bp in length with Cys 2. The sequence of the three oligonucleotide primers for amplification of cysteine proteinases is shown in figure 4.3.

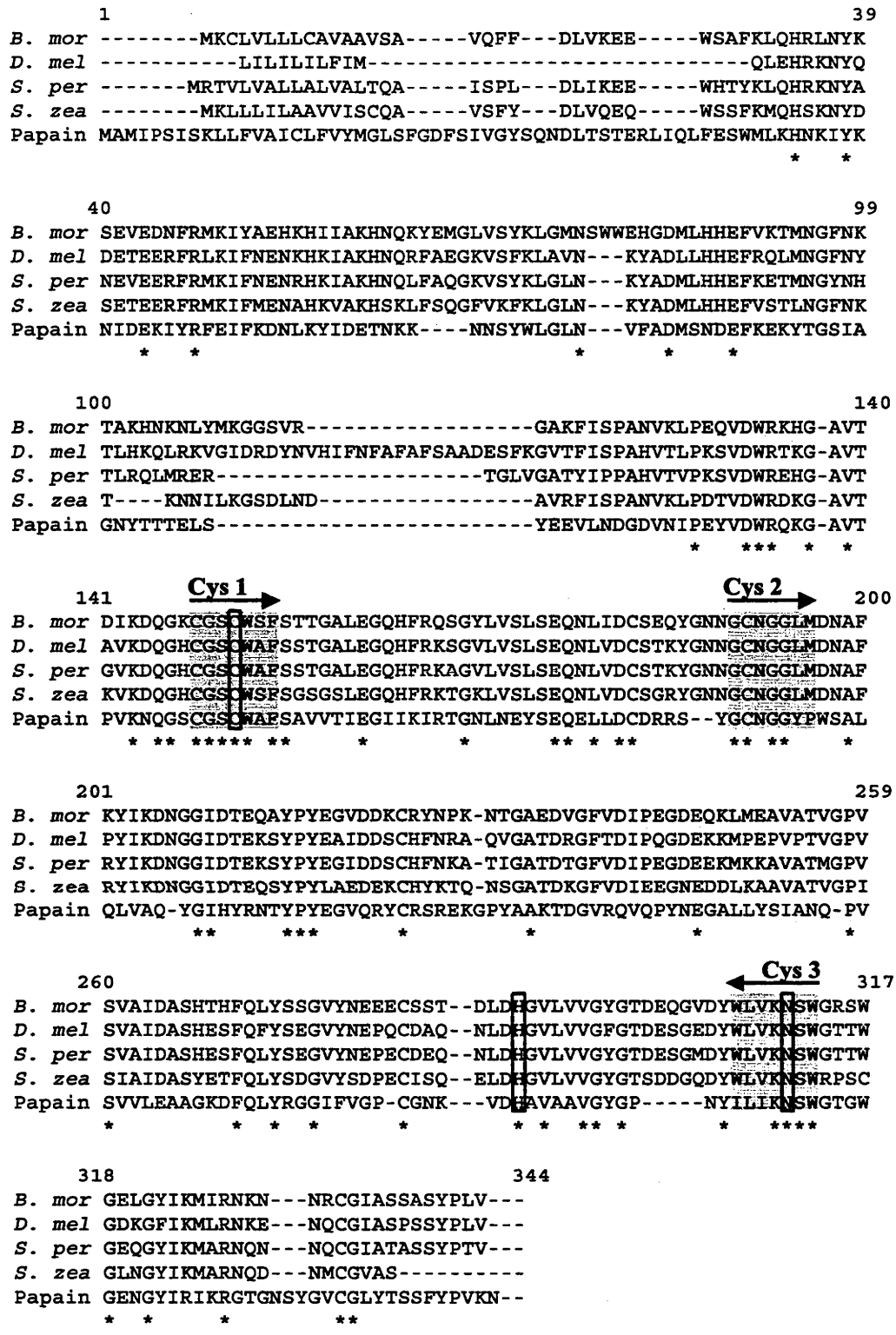

Figure 4.2: Alignment of the amino acid sequences of the insect cysteine proteinases, *Bombyx mori* cysteine proteinase (*B. mor*), *Drosophila melanogaster* cysteine proteinase 1 (*D. mel*), *Sarcophaga peregrina* pro-cathepsin L (*S. per*), *Sitophilus zeamais* cysteine proteinase *(S. zea)* and Papain (Papain). Numbering is according to the *B. mori* sequence. * under the alignment show identical residues. Degenerate primers used for PCR are shown as Cys x and are shaded. Active site residues are boxed, and dashes represent gaps introduced to maximise alignment.

	5'	3'
Cys 1:	TG(CT)GG(ACTG)(AT)(CG)(ACTG)TG(CT)TGGGC(ACTG)TT	
Cys 2:	GG(ACTG)TG(CT)AA(CT)GG(ACTG)GG(ACTG)(CT)T(ACTG)ATG	
Cys 3:	CCA(ACTG)(CG)(AT)(AG)TT(CT)TT(ACTG)AC(ACTG)A(AG)CCA	

Figure 4.3: Nucleotide sequences of the primers designed from cysteine proteinase sequences. Parentheses indicate degeneracy with the indicated bases present in equimolar amounts.

4.2.8.3 Serine Proteinase Primers

Degenerate primers, designed by I. Zolfaghar (1994) to the conserved regions of dipteran trypsin-like genes (Fig 4.4), were also used for RT-PCR. Four primers were designed to allow a fully nested PCR strategy. Tryp 1 and Tryp 4 are external primers which produce a 480 bp fragment in the *D. melanogaster* alpha trypsin sequence. Tryp 2 and Tryp 3 are internal primers which produce a fragment 396 bp long in the *D. melanogaster* alpha trypsin sequence. Tryp 1 and Tryp 3 prime in the sense orientation, and Tryp 2 and Tryp 4 prime in the antisense orientation. The DNA sequence of the four degenerate primers for amplification of serine proteinases is shown in Figure 4.5.

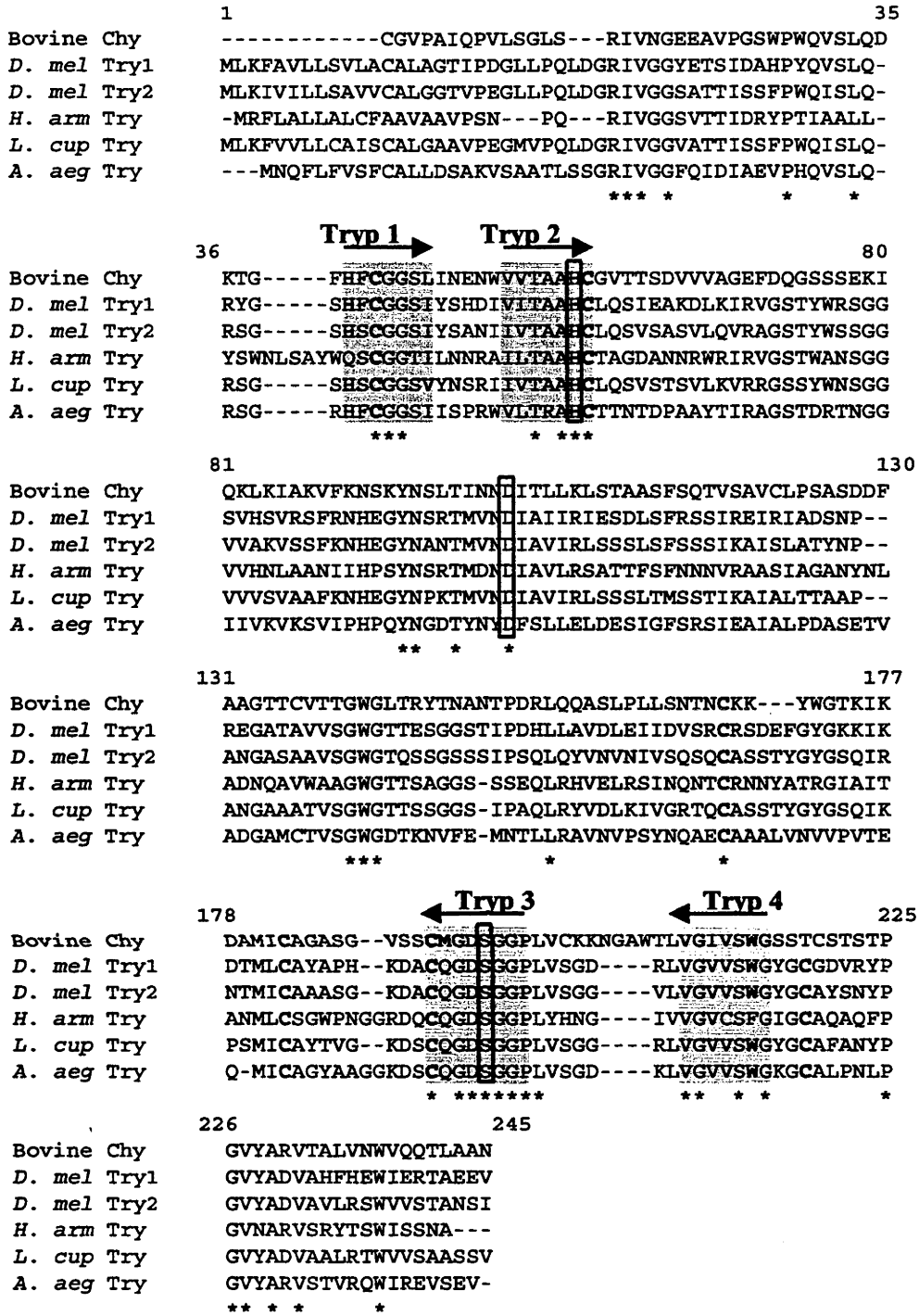

Figure 4.4: Alignment of amino acid sequences of Bovine chymotrypsin (Bovine Chy) with the insect sequences *Drosophila melanogaster* epsilon trypsin (*D. mel* Try1), *D. melanogaster* alpha trypsin, (*D. mel* Try2), *Helicoverpa armigera* trypsin (*H. arm* Try), *Lucilia cuprina* trypsin (*L. cup* Try), and *Aedes aegypti* 3A1 trypsin (*A. aeg* Try). Numbering is according to the bovine chymotrypsin system. * under the alignment show identical residues. Degenerate primers are shown as **TrypX** and are shaded. Catalytic triad residues of serine proteinases are boxed. Conserved cysteine residues are in bold, and dashes represent gaps introduced to maximise the alignment.

	5'	3'
Tryp 1	CA(CT)TT(CT)TG(CT)GGIGGI(TA)(CG)(ACGT)(ACT)T	
Tryp 2	(GA)TI(GA)TIAC(ACGT)GCIG(CG)ICA(CT)TG	
Tryp 3	GG(GTA)CCICCI(GC)ATAGTCICCCTTG(GA)CA	
Tryp 4	CCCCAI(GC)(AT)IACIA(CT)(GTA)CC(ACGT)AC	

Figure 4.5: Nucleotide sequences of the primers designed from serine proteinase sequences. Parentheses indicate degeneracy with the indicated bases present in equimolar amounts, and I denotes an inosine.

4.2.8.4 PCR reactions

RT-PCR was performed on mRNA prepared from mirid salivary gland and midgut tissues (see 4.2.7) using the Access RT-PCR System (Promega). Each reaction contained 10 µl of AMV/*Tfl* 5x reaction buffer, 1 µl dNTP mixture, 3 µl $MgSO_4$, 5 units AMV reverse transcriptase, 5 units *Tfl* DNA polymerase, 50 pmol of each primer, 2 µl (140 ng) mRNA template, and nuclease free water to 50 µl.

For isolation of cysteine proteinases, the primers used were Cys 1 and Cys 3, and the amplification conditions were 45°C for 45 min, 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 1 min, and 68°C for 2 min, followed by one cycle of 68°C for 7 min. Half-nested PCR was performed on 2 µl of the RT-PCR product, and included 5 µl of 10x Gibco BRL reaction buffer, 2 mM $MgSO_4$, 0.25 mM dNTP, 1.25 units Taq DNA polymerase, 50 pmol primer Cys 2, and 50 pmol primer Cys 3 in the reaction. The amplification conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 30 sec, followed by one cycle of 72°C for 7 min.

For isolation of serine proteinases, the primers used in the RT-PCR reaction were Tryp 1 and Tryp 4, and the amplification conditions were 45°C for 45 min, 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 45°C for 1 min, and 68°C for 2 min, followed by one cycle of 68°C for 7 min. Nested PCR was performed on 2 µl of the RT-PCR product, and included 5 µl of 10x Gibco BRL reaction buffer, 1.5 mM

$MgSO_4$, 0.25 mM dNTP, 1.25 units Taq DNA polymerase, 50 pmol primer Tryp 2, and 50 pmol primer Tryp 3 in the reaction. The amplification conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 45°C for 1 min, 72°C for 30 sec, followed by one cycle of 72°C for 7 min. All products were visualised on 1% agarose gels.

4.2.8.5 Cloning PCR products

PCR products were cut out of the agarose gels and purified using the BANDPURE Purification Kit (Progen), according to the manufacturer's instructions. Purified fragments were ligated into the pGEM®-T Easy plasmid vector using the pGEM®-T Easy Vector System (Promega).

4.2.9 Isolation of cDNA Clones

4.2.9.1 Construction of the cDNA library

Approximately 5 µg of mRNA prepared from total RNA (see 4.2.6) was used as a template to synthesise cDNA with EcoRI/NotI Adaptors, using the TimeSaver® cDNA Synthesis Kit (Pharmacia Biotech) according to the manufacturer's instructions. This yielded 140 µl of cDNA, and 15 µl of this was then ligated into λgt11 arms and packaged *in vitro* using a Packagene extract (Promega) as described by the manufacturer. Packaged phage was titred with Y1090 cells on Luria broth (LB) medium plates (Sambrook *et al.*, 1989) to determine the number of plaque forming units per ml.

4.2.9.2 Isolation of cDNA clones

All methods used for screening of the cDNA library were from Sambrook *et al.* (1989). Briefly, bacteriophage λgt11 were grown on a lawn of Y1090 cells at a concentration of approximately 50,000 plaque forming units per 15 cm LB plate. Phage DNA was replicated in duplicate onto nitrocellulose filters (Nitrobind, Micron Separations Inc.) and fixed by baking at 80°C for 2 hours in a vacuum oven. Filters were probed with PCR clone s621.2 (green mirid salivary gland serine proteinase),

prepared as described in section 4.2.5. Hybridisation was carried out at 65°C as described in section 4.2.3.2.

Hybridising clones were plaque purified as described in Sambrook *et al.* (1989), and used to make a high titre stock. Phage DNA was isolated from 100 ml liquid lysate as follows. Chloroform was added to 0.3% and the lysate shaken at 37°C for 30 min. The lysate was decanted from the chloroform and centrifuged at 16,000 *g* for 10 min. DNase I and RNase A were added to the supernatant to 1 µg/ml and 2 µg/ml respectively. The solution was then incubated at 37°C for 30 min and centrifuged at 131,000 *g* for 1 hour at 4°C. The phage pellet was resuspended in 1.2 ml of SM phage buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris.Cl pH7.5, 0.01% gelatin), and to this was added SDS to 0.5% (w/v), EDTA to 20 mM, and proteinase K to 50 µg/ml. The resuspended phage was incubated at 60°C for 1 hr with occasional aspiration, then extracted twice with equal volumes of phenol, and once with an equal volume of chloroform. The final supernatant was layered onto a salt cushion of 1 M NaCl in TE buffer and centrifuged at 341,000 *g* at 20°C for 2.5 hours. The supernatant was discarded and the phage DNA pellet was resuspended in 100 µl of TE buffer and left at 4°C.

The cloned DNA was liberated from the phage DNA by standard restriction enzyme digestion, ligated into pBC KS+ phagemid vector, and electroporated into DH10ß cells.

4.2.10 Sequence Analysis

Double stranded plasmid DNA was sequenced by the ABI PRISM™ Dye Terminator Cycle Sequencing Kit protocol (Perkin Elmer). Sequencing reactions were run on the Applied Biosystems Model 373 automated DNA sequencer, and sequence was analysed using the GCG software package (Genetics Computer Group, Program manual for theWisconsin package, Version 8, August 1994, Madison, WI). Database searches of non-redundant nucleotide databases (Genbank, EMBL, DNA Database of Japan (DDBJ), and Protein Data Bank (PBD)) and non-redundant protein databases (GenPept, SwissProt, Translation from EMBL (TREMBL), and Protein Information

Resource (PIR)) were performed using both Basic Local Alignment Search Tool (BLAST) programs, and FastA programs, through the Australian National Genomic Information Service (ANGIS) web site (http://www.angis.org.au). Multiple sequence alignments and similarity trees were constructed with the PILEUP algorithm of the GCG software package.

4.2.11 *In situ* Hybridisation

The method used is a modification of the non-radioactive tissue wholemount procedure described by Tautz and Pfeifle (1989).

4.2.11.1 Tissue Preparation

Salivary gland complexes and midguts were dissected from adult green mirids into phosphate buffered saline (PBS) (130 mM NaCl, 10 mM Sodium Phosphate, pH 7.2), then fixed in 4% PP (4% paraformaldehyde in PBS) for 1 - 2 hours. Tissues were treated in 90% Methanol/10% EGTA ethylene glycol-bis(β-aminoethyl ether) (EDTA) (ME) for five minutes, followed by passaging through the following steps; 7:3 ME:PP for 5 min, 1:1 ME:PP for 5 min, 3:7 ME:PP for 5 min, 100% PP for 20 min, and 100% PBS for 10 min. Fixed tissues were permeabilised by washing 3 x 5 min in PBT (PBS + 0.1% Tween 20), then incubating for 3 min in proteinase K (50 µg/ml in PBS). Digestion was stopped by incubating in 2 mg/ml glycine in PBT for 2 min. Tissues were then washed 2 x 5 min in PBT, refixed for 20 min in PP, and washed 3 x 10 min in PBT.

4.2.11.2 Probe Synthesis

DIG labelled ribo-probes were made from cDNA clones of the green mirid salivary gland serine proteinase gene *CdSp1* in both the sense and antisense orientation. pBC KS+ plasmid containing the insert was digested for 2.5 hours with XbaI, and to the completed digest reaction was added Proteinase K to 0.2 mg/ml and SDS to 0.5% (w/v). This was incubated at 37°C for 1 hour then extracted with an equal volume of phenol/chloroform and ethanol precipitated. After centifugation, pellets were

resuspended in 50 µl RNase free water, and 2 µl of each was used to synthesise DIG labelled RNA using reagents from the *AmpliScribe*™ T7 Transcription kit (Epicentre Technologies, except DIG-UTP from Boehringer Mannheim). The reaction contained 2 µl T7 reaction buffer, 1mM each of dATP, dGTP and dCTP, 0.65 mM dUTP, 0.35 mM DIG UTP, 2 µl DTT, and 2 µl T7 RNA polymerase solution (10 units of enzyme). The reaction was left at 37°C for 2 hours, then it was incubated with 1 µl RNase free DNase at 37°C for 15 min. The RNA was ethanol precipitated and resuspended in 50 µl RNase free water. To this was added 5.5 µl of 1 M Sodium carbonate pH 10.2, and this was incubated at 60°C for 30 min. The RNA was again ethanol precipitated, and the pellet resuspended in 50 µl RNase free water.

4.2.11.3 Hybridisation and Visualisation

Fixed and permeabilised tissues were prehybridised in hybridisation solution (50% formamide, 4 x SSC, 0.25 mg/ml tRNA, 0.5 mg/ml salmon sperm DNA, 50 µg/ml heparin, 0.1% Tween 20, 1 x Denhardt's solution, 5% dextran sulphate, sterile water to 22 ml) at 50°C for 1 hour before addition of 2 µl of probe. Tissues were hybridised at 50°C for 48 hours, rinsed twice with wash buffer (50% formamide, 4 x SSC, 0.1% Tween 20), then washed 4 times with wash buffer at 50°C for a total of 24 hours. Tissues were washed in PBT for 30 min, and incubated overnight at room temperature with anti-DIG-alkaline phosphatase antibody diluted 1/2000 in PBT. Tissues were then washed for 4 x 60 min with PBT, rinsed with AP buffer (100 mM Tris.Cl pH9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20) and washed for 5 min in AP buffer. Bound probe was visualised by adding 0.3 ml of AP buffer containing 2.7 µl NBT (75 mg/ml in dimethylformamide (DMF)) and 2.1 µl BCIP (50 mg/ml in DMF). When the desired colour development was achieved, tissues were rinsed three times with PBT and stored in 70% glycerol.

4.3 RESULTS

4.3.1 RT-PCR

Degenerate PCR primers were designed from conserved regions of cysteine proteinases (Fig. 4.2 and Fig. 4.3). Primers Cys 1 and Cys 3 were used in an RT-PCR reaction with mRNA from salivary gland and midgut tissues of the green mirid as the template. A fragment of approximately 500 bp in length was amplified from the midgut, but no detectable product was amplified from the salivary glands (Fig. 4.6 A). Half-nested PCR, using primers Cys 2 and Cys 3, was performed on the products of the RT-PCR reaction. A band of the expected size, approximately 380 bp in length, was amplified from the midgut, along with a second band of approximately 500 bp. Again no band was detected in the salivary glands (Fig. 4.6 B). The 380 bp midgut band was isolated and ligated into the vector pGEM®-T Easy. Ligated plasmids were electroporated into DH10ß *E.coli* cells, which were plated onto LB plates containing ampicillin, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) and isopropyl β-D-thiogalactoside (IPTG) for blue/white colony selection.

Degenerate PCR primers Tryp 1 and Tryp 4, designed against serine proteinase genes (Fig. 4.4 and Fig. 4.5), were also used in an RT-PCR reaction with mirid salivary gland and midgut mRNA as the templates. Some smeared DNA, with no obvious bands, was amplified from both the salivary gland mRNA and midgut mRNA (Fig. 4.7 A). Nested PCR with primers Tryp 2 and Tryp 3, performed on the RT-PCR products, yielded clear bands of the expected size, approximately 400 bp in length, from both the salivary gland and midgut (Fig. 4.7 B). Both bands were cloned as described above.

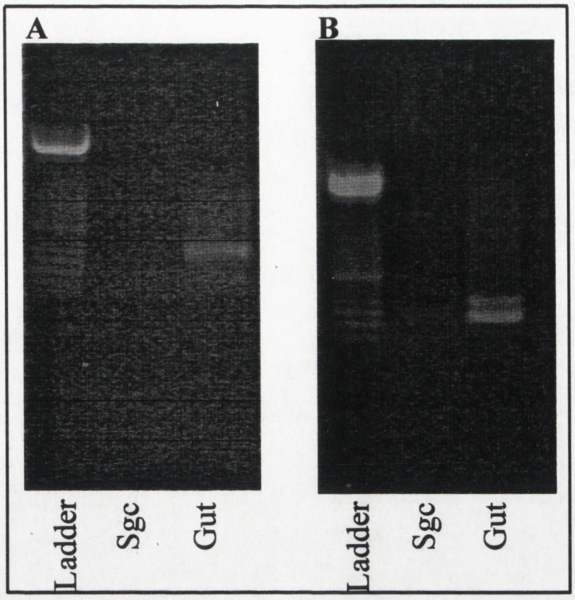

Figure 4.6: 1% agarose gels with 100 base pair (bp) ladder and amplicons from green mirid salivary gland mRNA (Sgc) and midgut mRNA (Gut) resulting from **A:** RT-PCR with cysteine proteinase primers Cys 1 and Cys 3, and **B:** Half nested PCR with cysteine proteinase primers Cys 2 and Cys 3.

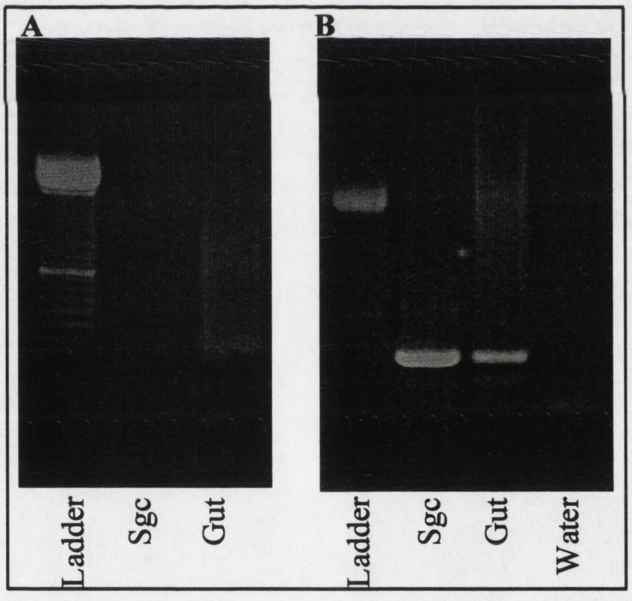

Figure 4.7: 1% agarose gels with 100 bp ladder and amplicons from green mirid salivary gland mRNA (Sgc) and midgut mRNA (Gut), plus a water control, resulting from **A:** RT-PCR with serine proteinase primers Tryp 1 and Tryp 4, and **B:** Nested PCR with serine proteinase primers Tryp 2 and Tryp 3.

4.3.2 Characterisation of Midgut Cysteine Proteinase Genes

Six independent clones containing the pGEM®-T Easy plasmid with the midgut cysteine proteinase fragment inserted were sequenced on both strands. Three different amplicons were identified, c63.2, c63.5, and c63.6 (Fig. 4.8 and Fig. 4.9). The sequence similarity between the amplicons is shown in Table 4.1.

To screen for the presence of more cysteine proteinase genes, plasmid DNA from a further 17 clones was digested, run on three agarose gels and blotted onto nylon membranes. Each membrane was probed with one of the mirid cysteine proteinase PCR amplicons (Fig. 4.10). c63.2 hybridised to 10 new clones, c63.5 hybridised to 1 new clone, and c63.6 hybridised to 5 new clones. One clone, c63.27, hybridised to none of the probes. This clone was subsequently sequenced and database searches established that is was most likely a fragment of a myosin gene (Appendix 6).

The three mirid cysteine proteinase sequences were used to search non-redundant nucleotide databases, and the predicted amino acid sequences were used to search non-redundant protein databases (Table 4.2 and Table 4.3). All three cysteine proteinase PCR amplicons showed substantial amino acid identity (56.3% - 65.3%) to a silkmoth cysteine proteinase and to cathepsin L proteinases from shrimp and fleshfly. In addition, all three amplicons were included in the construction of a similarity tree using the PILEUP algorithm of the GCG software package (Fig 4.11).

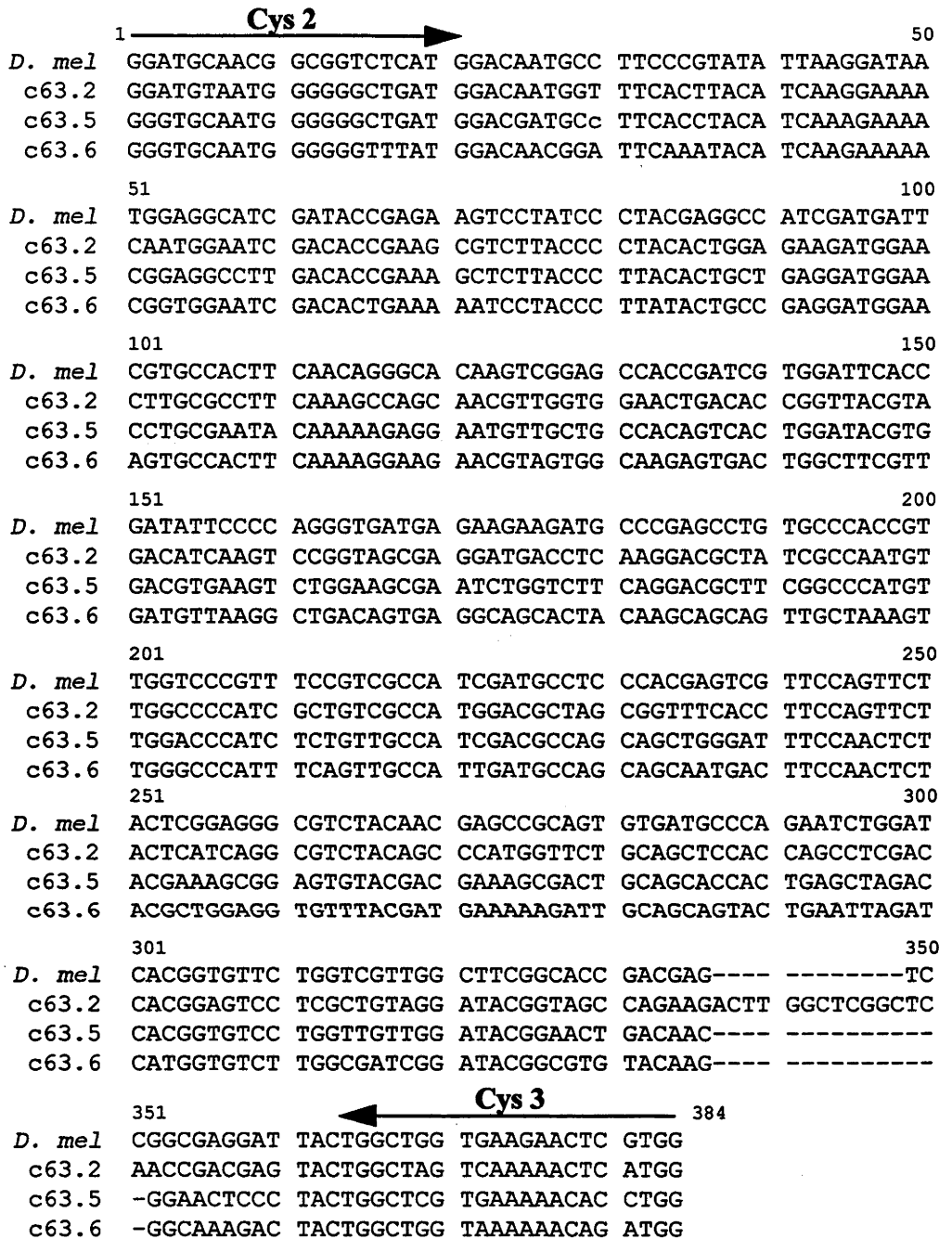

Figure 4.8: Nucleotide sequences of PCR amplicons amplified from green mirid midgut mRNA (c63.2, c63.5, c63.6) using degenerate cysteine proteinase primers, aligned with the corresponding region from *D. melanogaster* cysteine proteinase 1. The primers are shown as **Cys X.** →

```
          1                                                            50
P. van  ----MKFLTV LACVVAAAVA SPSLRQQWRD FKAEHGRRYA SVQEERYRLS
D. mel  ----LILILI LFIM------ ---------- -QLEHRKNYQ DETEERFRLK
S. per  MRTVLVALLA LVALTQAISP LDLIKEEWHT YKLQHRKNYA NEVEERFRMK
B. mor  -MKCLVLLLC AVAAVSAVQF FDLVKEEWSA FKLQHRLNYK SEVEDNFRMK
S. zea  -MKLLLLILAA VVISCQAVSF YDLVQEQWSS FKMQHSKNYD SETEERFRMK
 c63.2  ---------- ---------- ---------- ---------- ----------
 c63.5  ---------- ---------- ---------- ---------- ----------
 c63.6  ---------- ---------- ---------- ---------- ----------

          5                                                           100
P. van  VFEQNQQFID DHNARFENGE VTFTLQMNQF ---GDMTSEE FTATMNGFLN
D. mel  IFNENKHKIA KHNQRFAEGK VSFKLAVNKY ---ADLLHHE FRQLMNGFNY
S. per  IFNENRHKIA KHNQLFAQGK VSYKLGLNKY ---ADMLHHE FKETMNGYNH
B. mor  IYAEHKHIIA KHNQKYEMGL VSYKLGMNSW WEHGDMLHHE FVKTMNGFNK
S. zea  IFMENAHKVA KHSKLFSQGF VKFKLGLNKY ---ADMLHHE FVSTLNGFNK
 c63.2  ---------- ---------- ---------- ---------- ----------
 c63.5  ---------- ---------- ---------- ---------- ----------
 c63.6  ---------- ---------- ---------- ---------- ----------

          101                                                         150
P. van  VPSRRPTAI- ---------- ---------- -------LRA DPDETLPKEV
D. mel  TLHKQLRKVG IDRDYNVHIF NFAFAFSAAD ESFKGVTFIS PAHVTLPKSV
S. per  TLRQLMR--- ---------- --------ER TGLVGATYIP PAHVTVPKSV
B. mor  TAKHNKNLYM KGGSVR---- ---------- ---GA-KFIS PANVKLPEQV
S. zea  T----KNNIL KGSDLN---- ---------- ---DAVRFIS PANVKLPDTV
 c63.2  ---------- ---------- ---------- ---------- ----------
 c63.5  ---------- ---------- ---------- ---------- ----------
 c63.6  ---------- ---------- ---------- ---------- ----------

                                Cys1 ▶
          151                                                         200
P. van  DWRTKGAVTP VKDQKQCGSC WAFSTTGSLE GQHFLKDGKL VSLSEQNLVD
D. mel  DWRTKGAVTA VKDQGHCGSC WAFSSTGALE GQHFRKSGVL VSLSEQNLVD
S. per  DWREHGAVTG VKDQGHCGSC WAFSSTGALE GQHFRKAGVL VSLSEQNLVD
B. mor  DWRKHGAVTD IKDQGKCGSC WSFSTTGALE GQHFRQSGYL VSLSEQNLID
S. zea  DWRDKGAVTK VKDQGHCGSC WSFSGSGSLE GQHFRKTGKL VSLSEQNLVD
 c63.2  ---------- ---------- ---------- ---------- ----------
 c63.5  ---------- ---------- ---------- ---------- ----------
 c63.6  ---------- ---------- ---------- ---------- ----------

          Cys2 ▶
          201                                                         250
P. van  CSDKFGNMGC MGGLMDQAFR YIKANKGIDT EDSYPYEAQD GKCRFDASNV
D. mel  CSTKYGNNGC NGGLMDNAFP YIKDNGGIDT EKSYPYEAID DSCHFNRAQV
S. per  CSTKYGNNGC NGGLMDNAFR YIKDNGGIDT EKSYPYEGID DSCHFNKATI
B. mor  CSEQYGNNGC NGGLMDNAFK YIKDNGGIDT EQAYPYEGVD DKCRYNPKNT
S. zea  CSGRYGNNGC NGGLMDNAFR YIKDNGGIDT EQSYPYLAED EKCHYKTQNS
 c63.2  ---------- -----DNGFT YIKENNGIDT EASYPYTGED GTCAFKASNV
 c63.5  ---------- -----DDAFT YIKENGGLDT ESSYPYTAED GTCEYKKRNV
 c63.6  ---------- -----DNGFK YIKKNGGIDT EKSYPYTAED GKCHFKRKNV

          251                                                         300
P. van  GATDTGYVDV EHGSESALKK AVATIGPISV AIDASQPSFQ FYHDGVYYEE
D. mel  GATDRGFTDI PQGDEKKMPE PVPTVGPVSV AIDASHESFQ FYSEGVYNEP
S. per  GATDTGFVDI PEGDEEKMKK AVATMGPVSV AIDASHESFQ LYSEGVYNEP
B. mor  GAEDVGFVDI PEGDEQKLME AVATVGPVSV AIDASHTHFQ LYSSGVYNEE
S. zea  GATDKGFVDI EEGNEDDLKA AVATVGPISI AIDASYETFQ LYSDGVYSDP
 c63.2  GGTDTGYVDI KSGSEDDLKD AIANVGPIAV AMDASGFTFQ FYSSGVYSPW
 c63.5  AATVTGYVDV KSGSESGLQD ASAHVGPISV AIDASSWDFQ LYESGVYDES
 c63.6  VARVTGFVDV KADSEAALQA AVAKVGPISV AIDASSNDFQ LYAGGVYDEK
```

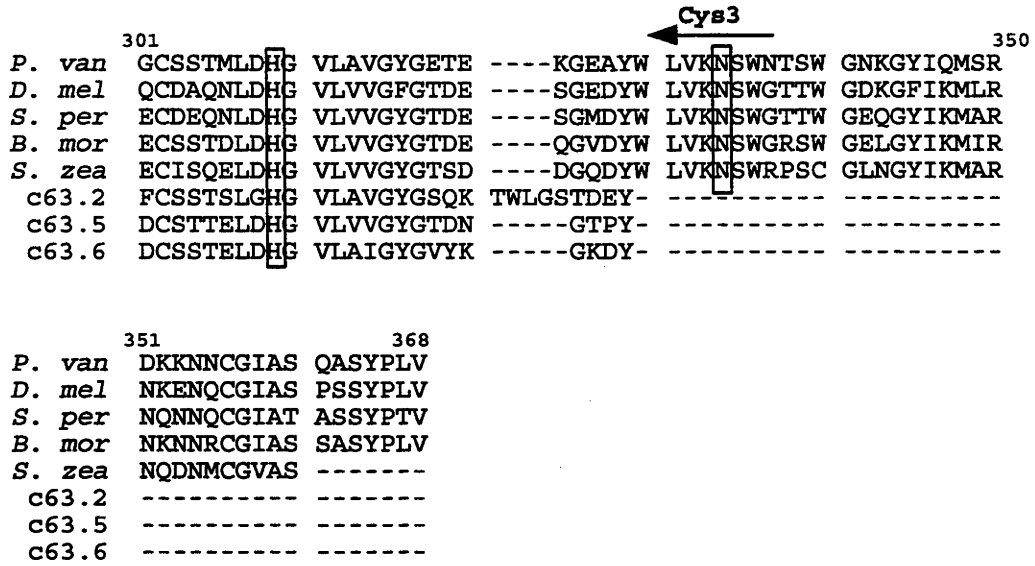

Figure 4.9: Alignment of the predicted amino acid sequences of the three cysteine proteinase PCR amplicons from the midgut of the green mirid (c63.2, c63.5 and c63.6), with *Penaeus vannamei* cathepsin L (*P. van*), *Drosophila melanogaster* cysteine proteinase 1 (*D. mel*), *Sarcophaga peregrina* pro-cathepsin L (*S. per*), *Bombyx mori* cysteine proteinase (*B. mor*), and *Sitophilus zeamais* cysteine proteinase (*S. zea*). Primer sequences are not included in the amino acid translation of the mirid amplicons. Cys X indicates degenerate primers used in isolation of the clones. The active site residues are boxed, and dashes represent gaps introduced to maximise the alignment.

Table 4.1: Sequence similarity between mirid cysteine proteinase PCR amplicons.

	Nucleotide Identity (%)	Amino Acid Identity (%)	Amino Acid Similarity (%)
c63.6/c63.5	71.8	71.5	81.3
c63.6/c63.2	64.8	64.2	74.8
c63.5/c63.2	70.7	67.5	79.7

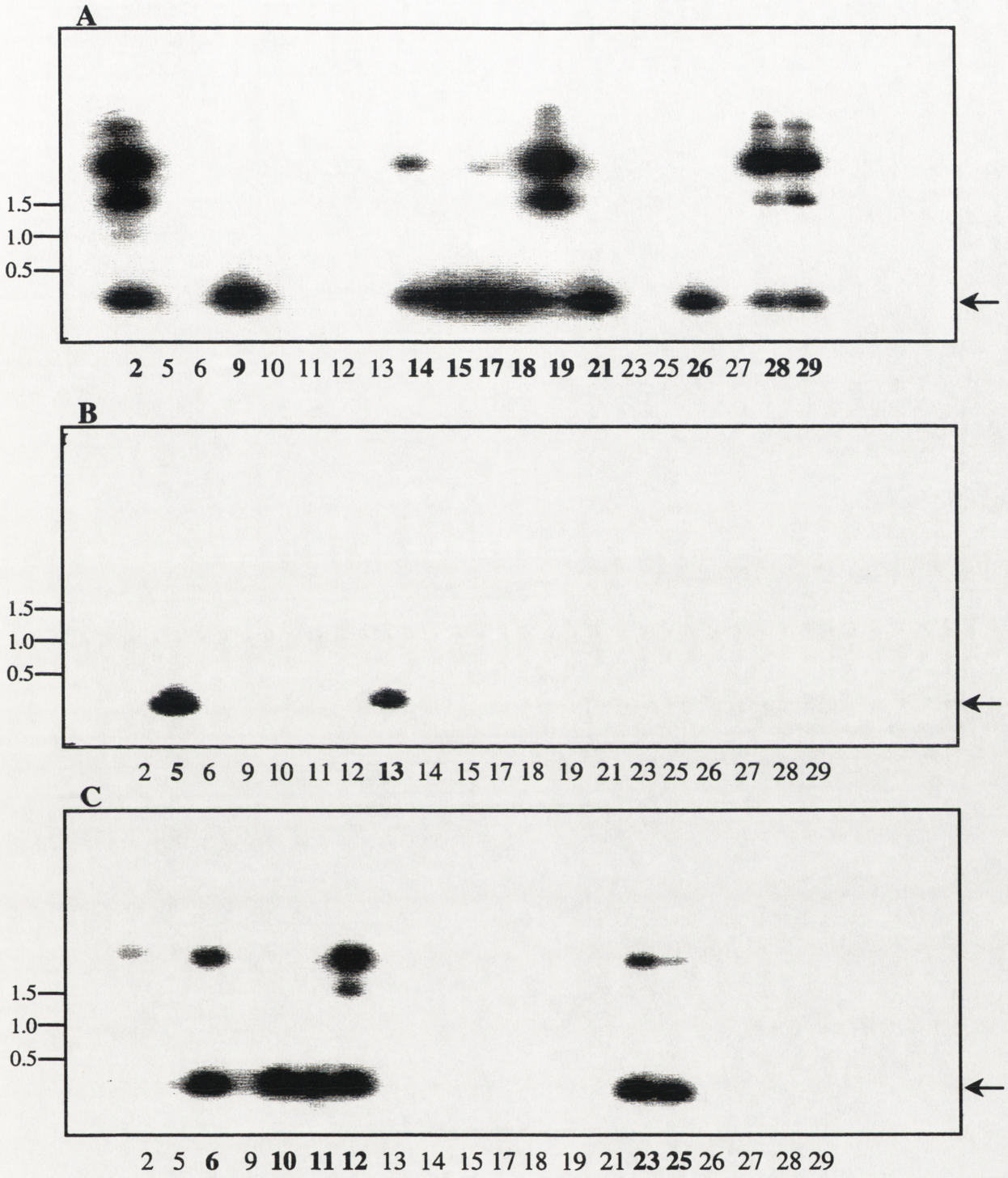

Fig 4.10: Southern blots of green mirid cysteine proteinase clones, probed with PCR amplicons **A**: c63.2, **B**: c63.5, and **C**: c63.6. Numbers underneath refer to clone numbers, and those in bold hybridised to the probe used. Numbers on the left show approximate size in kb, and arrows indicate proteinase amplicons.

Table 4.2: Most similar nucleotide (nt) sequences to mirid cysteine proteinase PCR amplicons, determined by FastA searching of non redundant nucleotide databases.

Clone	Homologous sequence	% identity
c63-2	*Penaeus vannamei* (shrimp) cathepsin-L gene	66.5% over 337 nt
	Sitophilus zeamais (maize weevil) cysteine proteinase	66.8% over 334 nt
	Bombyx mori (silkmoth) cysteine proteinase	64.7% over 329 nt
c63-5	*P. vannamei* cathepsin-L like cysteine proteinase	65 1% over 372 nt
	B. mori cysteine proteinase	64.5% over 372 nt
	Homarus americanus (lobster) cysteine proteinase	64.0% over 369 nt
c63-6	*S. zeamais* cysteine proteinase	66.6% over 365 nt
	B. mori cysteine proteinase	63.7% over 375 nt
	P. vannamei cathepsin-L like cysteine proteinase	63.1% over 374 nt

Table 4.3: Most similar sequences to the predicted amino acid (aa) sequences of mirid cysteine proteinase PCR amplicons, determined by FastA searching of non redundant protein databases.

Clone	Homologous sequence	% identity
c63.2	*P.vannamei* cathepsin L	63.3% over 128 aa
	Sarcophaga peregrina (fleshfly) cathepsin L	56.3% over 128 aa
	B.mori cysteine proteinase	57.8% over 128 aa
c63.5	*B. mori* cysteine proteinase	62.9% over 124 aa
	P.vannamei cathepsin L	65.3% over 124 aa
	S. peregrina cathepsin L	60.5% over 124 aa
c63.6	*B. mori* cysteine proteinase	62.9% over 124 aa
	P.vannamei cathepsin L	65.3% over 124 aa
	S. peregrina cathepsin L	59.7% over 124 aa

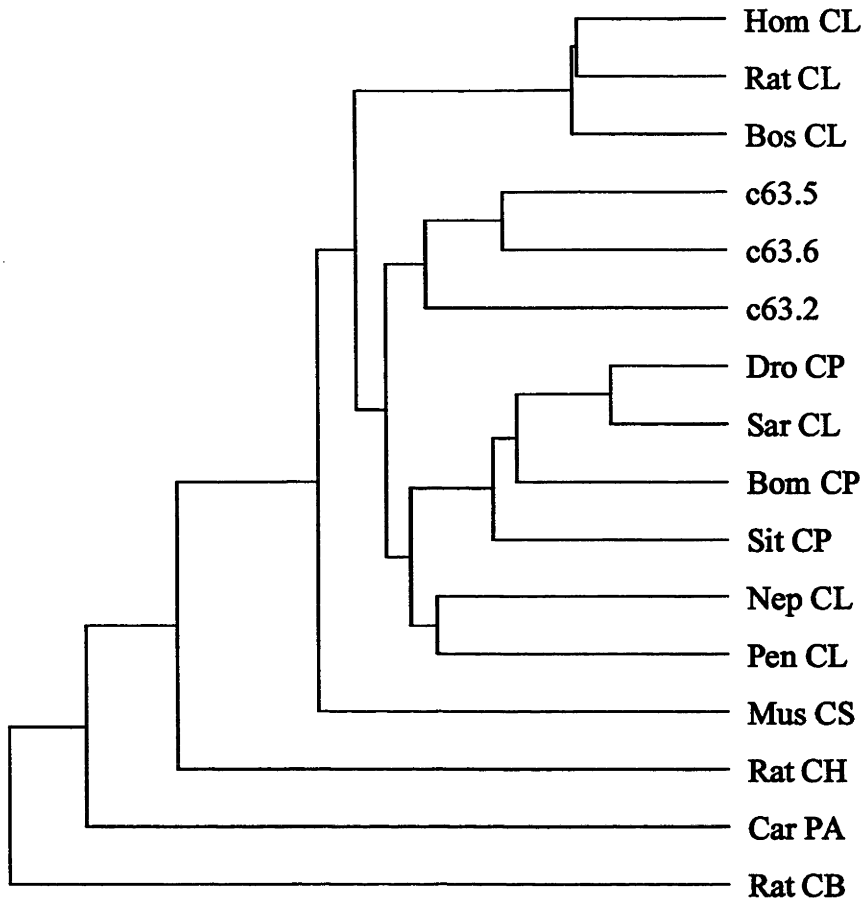

Figure 4.11: Mirid midgut cysteine proteinase protein similarity tree. Tree was constructed with the Pileup function of the GCG software package (Genetics Computer Group). The following sequences were included, with accession numbers in brackets. Hom CL = *Homo sapiens* (human) cathepsin L (P07711); Rat CL = *Rattus norvegicus* (rat) cathepsin L (P07154); Bos CL = *Bos taurus* (bovine) cathepsin L (P25975); c63.5, c63.6, c63.2 = *Creontiades dilutus* cysteine proteinase PCR amplicons; Dro CP = *Drosophila melanogaster* (fruitfly) cysteine proteinase 1 (S67481); Sar CL = *Sarcophaga peregrina* (fleshfly) cathepsin L (A52810); Bom CP = *Bombyx mori* (silkmoth) cysteine proteinase (S77508); Sit CP = *Sitophilus zeamais* (maize weevil) cysteine proteinase (O46030); Nep CL = *Nephrops norvegicus* (Norway lobster) cathepsin L (S47433); Pen CL = *Penaeus vanameii* (shrimp) cathepsin L (S53027); Mus CS = *Mus musculus* (mouse) cathepsin S (O54973); Rat CH = *R. norvegicus* cathepsin H (P00786); Car PA = *Carica papaya* (papaya) papain (M15203); Rat CB = *R. norvegicus* cathepsin B (P00787)

4.3.3 Characterisation of Midgut Serine Proteinase Genes

Six independent clones containing the pGEM®-T Easy plasmid with the midgut serine proteinase fragment inserted were sequenced on both strands. The sequences of all 6 amplicons were essentially identical, differing from each other by 1 to 12 nucleotides over the 460 bp of sequence (Fig. 4.12). Further analysis was continued on a single green mirid midgut serine proteinase amplicon, s622.3 (Fig. 4.13 and Fig. 4.14).

To screen for the presence of more midgut serine proteinase genes, plasmid DNA from a further 20 clones was digested, run on an agarose gel and Southern blotted onto nylon membrane. The membrane was probed with amplicon s622.3, which hybridised to all clones (Fig. 4.15 A).

The sequence of s622.3 was used to search non-redundant nucleotide and protein databases (Table 4.4 and Table 4.5), and showed strong amino acid identity (35.7% to 39.4%) to dipteran trypsin-like proteinases.

4.3.4 Characterisation of Salivary Gland Serine Proteinase Genes

Nine independent clones containing the pGEM®-T Easy plasmid with the salivary gland serine proteinase fragment inserted were sequenced on both strands. From these, two different serine proteinase amplicons were identified, s621.2 and s621.4 (Fig. 4.13 and Fig. 4.14). Plasmid DNA from a further 19 clones was digested, run on two agarose gels and Southern blotted onto nylon membranes. Each membrane was probed with one of the mirid serine proteinase PCR amplicons (Fig. 4.15 B and Fig. 4.15 C). s621.2 hybridised to 2 new clones, and s621.4 hybridised to 16 new clones. One clone, s621.17, hybridised to neither probe, and so was subsequently sequenced and found to be another serine proteinase gene (Fig. 4.13 and Fig. 4.14).

All three salivary gland amplicons were used to search non-redundant nucleotide and protein databases (Table 4.4 and Table 4.5). s621.2 showed strong amino acid identity to both human chymotrypsin (32.5%) and to dipteran serine proteinase genes (approx. 30%). s621.4 showed significant amino acid identity (33.3% to 35.7%) to trypsin-like proteinases from a range of organisms. s621.17 showed

significant identity (29 to 30.8%) to limulus clotting factor from the horseshoe crab, to enteropeptidases from cow and pig, and to crayfish trypsins (Table 4.5). The similarities between the salivary serine proteinase amplicons and the midgut amplicon are shown in Table 4.6. All serine proteinase amplicons were also included in the construction of a similarity tree (Fig. 4.16).

```
          1                                                          60
s622.1    GTGGTGACGG CGGGGCATTG TGTCATAGGG ACAAGCCGGA AACTATACGT TGTTGCTGGT
s622.3    GTGGTGACGG CGGGGCATTG TGTCATAGGG ACAAGCCGGA AACTATACGT TGTTGCTGGT
s622.6    GTGGTGACGG CGGCGCATTG CGTCATAGGG ACAAGCCGAA GACTATACGT TGTTGCTGGT
s622.4    GTGGTGACGG CGGGGCATTG TGTCATAGGG ACAACCCGAA GACTATACGT TGTTGCTGGT
s622.7    ATGGTGACGG CGGGGCACTG TGTCATAGGG ACAAGCCGGA GACTATACGT TGTTGCTGGT
s622.2    GTGGTGACGG CGGCGCATTG TGTCATAGGG ACAAGCCGAA GACTATACGT TGTTGCTGGT

          61                                                        120
s622.1    GTGGATAGAA TAGACACTGG TGTACTGAAC CCAGTGGTTA TGGCTGTCGT ACATAAAGAC
s622.3    GTGGATAGAA TAGACACTGG TGTACTGAAC CCAGTGGTTG TGGCTGTCGT ACATAAAGAC
s622.6    GTGGATAGAA TAGACACTGG TGTACTGAAC CCAGTGGTTG TGGCTGTCGT ACATAAAGAC
s622.4    GTGGATAGAA TAGACACTGG TGTACTGAAC CCAGTGGTTG TGGCTGTCGT ACATAAAGAC
s622.7    GTGGATAGAA TAGACACTGG TGTACTGAAC CCAGTGGTTG TGGCTGTCGT ACATAAAGAC
s622.2    GTGGATAGAA TAGACACTGG TGTACTGAAC CCAGTGGTTG TGGCTGTCGT ACATAAAGAC

         121                                                        180
s622.1    TATGACAACA CAACAAACGA GAATGATTTG GCTGTGTTAC ACGTGAGTCG CGATTTGAAT
s622.3    TATGACAACA CAACAAACGA GAATGATTTG GCTGTGTTAC ACGTGAGTCG CGATTTGAAT
s622.6    TATGACAATA CAACAAACGA GAATGATTTG GCTGTATTAC ACGTGAGTCG CGATTTGAAT
s622.4    TATGACAACA CAACAAACGA GAATGATTTG GCTGTGTTAC ACGTGAGTCG CGATTTAAAT
s622.7    TATGACAACA CAACAAACGA GAATGATTTG GCTGTGTTAC ACGTGAGTCG CGATTTGAAT
s622.2    TATGACAACA CAACAAACGA GAATGATGTG GCTGTGTTAC ACGTGAGTCG CGATTTGAAT

         181                                                        240
s622.1    CTAGATAATA AAACCAGGAA AGCTCTTCCG ATCCCGACAA AAGTTCCTCT GGACGGGACA
s622.3    CTAGATAATA AAACCAGGAA AGCTCTTCCG ATCCCGACAA AAGTTCCTCT GGACGGGACA
s622.6    CTAGATAATA AAACCAGGAA AGCCCTTCCG ATCCCGACAA AAGTTCCTCA GGTCGGGACA
s622.4    CTAGATAATA AAACCAGGAA AGCTCTTCCG ATCCCGACAA AAGTTCCTCT GGACGGGACA
s622.7    CTAGATAATA AAACCAGGAA AGCTCTTCCG ATCCCGACAA AAGTTCCTCT GGACGGGACA
s622.2    TTAGATAATA AAACCAAGAA AGCCCTTCCG ATCCCGACAA AAGTTCCTCA GGACGGGACA

         241                                                        300
s622.1    TTGTGCAGAG TTAGTGGCTG GGGCTCAACA GTCTTT---- GACCCCGTTA ATCCTCAGCC
s622.3    TTGTGCAGAG TTAGTGGCTG GGGCTCAACA GTCTTT---- GACCCCGTTA ATCCTCAGCC
s622.6    TTGTGCAGAG TTAGTGGCTG GGGCTCAACA GTCTTT---- GACCCCGTTA ATCCTCAGCC
s622.4    TTGTGCAGAG TTAGTGGCTG GGGCTCAACA GTCTTTGTAA GACCCCGTTA ATCCTCAGCC
s622.7    TTGTGCGGAG TTAGTGGCTG GGGCTCAACA GTCTTTGTAA GACCCCGTTA ATCCTCAGCC
s622.2    TTATGCAGAG TTAGTGGCTG GGGCTCAACA GTCTTTGTAA GACCCCGTTA ATCCTCAGCC

         301                                                        360
s622.1    TTCCAGAATT TTGCTAATGA CTGACGTACC CTTGTACAAC TTGGGGAAAT GCCGTCTTGA
s622.3    TTCCAGAATT TTGCTAATGA CTGACGTACC CTTGTACAAC TTGGGGAAAT GCCGTCTTGA
s622.6    TTCCAGAAAT TTGCTAATGA CGGTCGTACC CTTGTACAAC TTGGGGAAAT GCCGTCTTGA
s622.4    TTCCAGAAAT TTGCTAATGA CGGTCGTACC CTTGTACAAC TTGGGGAAAT GCCGTCTTGA
s622.7    TTCCAGAATT TTGCTAATGA CTGACGTACC CTTGTACAAC TTGGGGAAAT GCCGTCTTGA
s622.2    TTCCAGAATT TTGCTAATGA CTGACGTACC CTTGTACAAC TTGGGGAAGT GCCGTCGCGA

         361                                                        418
s622.1    ACTCGATGTG CCAATTGGCC AGATTTGTGC TGGCTACGAA GATGGAGGCC ATGATTCA
s622.3    ACTCGATGTG CCAATTGGCC AGATTTGTGC TGGCTACGAA GATGGAGGCC ATGATTCA
s622.6    ACTCGATGTG CCAATTGGCC AGATTTGTGC TGGCTACGAA GATGGAGGCC ATGATTCA
s622.4    ACTCGATGTG CCAATTGGCC AGATTTGTGC TGGCTACGAA GATGGAGGCC ATGATTCA
s622.7    ACTCGATGTG CCAATTGGCC AGATTTGTGC TGGCTACGAA GATGGAGGCC ATGATTCA
s622.2    ACTCGATGTG CCAATTGGCC AGATTTGTGC TGGCTACGAA GATGGAGGCC ATGATTCA
```

Figure 4.12: Nucleotide sequences of PCR products amplified from green mirid midgut mRNA using the degenerate serine proteinase primers Tryp 2 and Tryp 3. Nucleotides which differ between the sequences are shaded, and dashes represent gaps introduced to maximise the alignment.

```
          1                                                          60
 D. mel   CCATCTACTC TGCCAACATC ATTGTGACCG CCGCTCACTG ---------- ----------
 s621.2   ---------- ---------- GTGGTGACGG CGGGGCACTG ---------- ----------
 s621.4   ---------- ---------- GTGGTGACGG CGGGGCACTG ---------- ----------
s621.17   ---------- ---------- ATGGTGACGG CGGCGCACTG TGTCAGTGAC CGTTGGACCA
 s622.3   ---------- ---------- GTGGTGACGG CGGGGCATTG ---------- ----------

         61                                                         120
 D. mel   ---------- ---------- CTGCAGTCCG TGTCCGCTTC AGTCCTGCAG GTCCGTGCTG
 s621.2   CACTGATGAC ATCATCAAGG CCAGA-ACCA GGACCGCTGT TCTTTTGGGA TCTCACGATC
 s621.4   -------CAA ACCAGAACGC AATGAGCCCC TGTCTGTTGT TCTCGCCGAA CACAAAGTCA
s621.17   AGGAGCGCAC CGATCCTTCC GATGTCGCTC CCGTTGTCGG AGCTCATAGC ACTTCACAAG
 s622.3   ---------- ---------- --TGTCATAG GGACAAGCCG GAAACTATAC GTTGTTGCTG

         121                                                        180
 D. mel   GATCCACCTA CTGGAGCTCT GGTGGCGTCG TCGCCAAGGT TTCCTCTTTC AAGAACCACG
 s621.2   GCTCACGTCC CAGCTCTACT GCTGTTACCA TCAACGTAGA AAGGATCAAC CAGCATGAAA
 s621.4   GCTCAAAAAC TGAGAGCAAG ACCACCATCA TTGATGTCAC CCGCATGATC ACTCACGAGC
s621.17   -GATTCTTCC CACGGATCAA CTCAAAAACT GGAGGTCAGA GAGATCATCA TTGCTGACTC
 s622.3   GTGTGGATAG AATAGACACT GGTGTACTGA ACCCAGTGGT TGTGGCTGTC GTACATAAAG

         181                                                        240
 D. mel   AGGGATACAA CGCTAACACC ATGGTCAACG ACATCGCTGT CATCCGTCTG AGCTCTTCCC
 s621.2   AATACAA--- CGCCAACACC ATCGCCAACG ACATTTCCAT CCTTACTCTC GCCAGTTCCA
 s621.4   AGTACGATCT GAAGAAAAAC ACCGAAAACG ACGTTGCTCT GTTGGTCCTT GCTAGCAAAA
s621.17   ATGGTTTGAT GCCAAAGGAA GTTTGAAGGG AGATATTGCA ATTATCGTAT TGAAAGAACC
 s622.3   ACTATGA--- CAACACAACA AACGAGAATG ATTTGGCTGT GTTACACGTG AGTCGCGATT

         241                                                        300
 D. mel   TGAGCTTCAG CTCAAGCATC AAGGCTATTA GCCTGGCCA- --CTTACAAC CCAGCTAACG
 s621.2   TCAACTTCAA CAAGGTCATC GGACCAGTTT GCTTGCCACT TCCAGGTCTT GACGTTAGCG
 s621.4   TCCCCTTCGG CAGAACTATT GGACCCGCCT GTTTCCCTAA GGCTAACCTC AACATTGTTG
s621.17   TATGAAGTAC AGCAAATGGG TCAGTCCAGT TTGTCTCACA CCGACTCAAC CTGATGTTGT
 s622.3   TGAATCTAGA TAATAAAACC AGGAAAGCTC TTCCGATCCC GACAAAAGTT CCTCTGGACG

         301                                                        360
 D. mel   GAGCCTCTGC CGCCGTTTCC GGTTGGGGTA CCCAGTCGTC CGGATCCAGC TCCATCCCCT
 s621.2   GACAGACTGT CAGAGTTCTT GGTTGGGGAG CTGAGAGGTT CCA---GGGT GCCATGACTA
 s621.4   GACAGAAAGT CCGTGTTATT GGATGGGGAG CACTCGCCTC AGG---TGGC AGACAGCCCG
s621.17   AGACAAATAC ATCAGGATGA TGGGATGGGG AGCAACTGGA GACGGATATC CCGACATTTT
 s622.3   GGACATTGTG CAGAGTTAGT GGCTGGGGCT CAACAGTCTT TGACCCCGTT ---AATCCTC

         361                                                        420
 D. mel   CTCCCAGCTG CAGTACGTGA ACGTGAACAT CGTTAGCCAG AGCCAGTGTG CTTCCTCCAC
 s621.2   TGAGGCCCAA GAAGCTGGAC ACTACCGCAG TCTCTCCTGC TCAATGTGCT GCTATCTGGC
 s621.4   ACATCCTCCA GAAGGTCGA- ---------- -TCTTGATGT TCAGCCCACC TCTGCCTGCT
s621.17   AAGGGAAGCT AATGCTCTCG TCATTAGCCC GAAAGACTGT AACAACGATG TCAAGGGCGA
 s622.3   AGCCTTCCAG AATTTTGCTA ATGACTGACG TACCCTTGTA CAACTTGGGG AAATGCCGTC

         421                                                        480
 D. mel   ACGGATACGG TAGCCAGATC CGCAACACCA TGATCTGCGC TGCTGCCAGC GGCAAGGATG
 s621.2   GTGGATTGGT GTCAGCCACC AACCCAACCC AAGTGTGCAC CCTTTCAAAG AAAGAGACCG
 s621.4   CCAGGGTCTA CAAAGGAATC ACTGAAGGCC AACTCTGCAC TTACACCCTC AGGAAAGACG
s621.17   CTATGCGGCG GTCCAATCAC TAGTGAATTC GCGGCCGCCT GCAGGTCGAC CATATGGGAG
 s622.3   TTGAACTCGA TGTGCCAATT GGCCAGATTT GTGCTGGCTA CGAAGATGGA GGCCATGATT

       481                                                          540
 D. mel   CCTGCCAGGG TGACTCCGGT GGCCCACTGG TCTCCGGCGG AGTCCTCGTC GGTGTTGT--
 s621.2   CCTGCCAAGG GCGACTATGC GGCGGCCC-- ---------- ---------- ----------
 s621.4   CTTGCCAAGG GCGACTATGC GGCGGCCC-- ---------- ---------- ----------
s621.17   AGCTCCCAAC GCG------- ---------- ---------- ---------- ----------
 s622.3   CATGCCAAGG GCGACTATCC GGCGGTCC-- ---------- ---------- ----------
```

Figure 4.13: Nucleotide sequences of PCR products amplified from green mirid salivary gland mRNA (s621.2, s621.4 and s621.17) and midgut mRNA (s622.3) using the degenerate serine proteinase primers Tryp 2 and Tryp 3, aligned with the corresponding region of the *D. melanogaster* alpha trypsin gene. Dashes represent gaps introduced to maximise the alignment.

```
           1                                                  50
A. aeg     ---------- ---------- ---------- --MNQFLFVS FCALLDSAKV
A. gam     ---------- ---------- ---------M SNKIAILLAV LVAVVACAEA
D. mel     ---------- ---------- ---------- MLKIVILLSA VVCALGGTVP
H. arm     ---------- ---------- ---------- -MRFLALLAL CFAAVAAVPS
S. per     LWALTQPAYV YEKPRDVVLL NQNTVAENFG QFISNYGADA DEEVIAYNEQ
Bov Chy    ---------- ---------- ---------- -----CGVPA IQPVL-----
s621.2     ---------- ---------- ---------- ---------- ----------
s621.4     ---------- ---------- ---------- ---------- ----------
s621.17    ---------- ---------- ---------- ---------- ----------
s622.3     ---------- ---------- ---------- ---------- ----------

           51                                                 100
A. aeg     SA-------- --ATLS---- ---SGRIVGG FQIDIAEVPH QVSLQRSG--
A. gam     QANQRHRLVR PSPSFSPRPR YAVGQRIVGG FEIDVSDAPY QVSLQYNK--
D. mel     EGLLPQ---- ---------- --LDGRIVGG SATTISSFPW QISLQRSG--
H. arm     NP-------- ---------- ----QRIVGG SVTTIDRYPT IAALLYSWNL
S. per     TALAKSEPRR KECSSKCFCG TPNVNRIVGG TQVRQNKYPW TAQLVKGRHY
Bov Chy    ---------- ---------- -SGLSRIVNG EEAVPGSWPW QVSLQDKTGF
s621.2     ---------- ---------- ---------- ---------- ----------
s621.4     ---------- ---------- ---------- ---------- ----------
s621.17    ---------- ---------- ---------- ---------- ----------
s622.3     ---------- ---------- ---------- ---------- ----------

                Tryp1 →          Tryp2 →
           101                                                150
A. aeg     ---RHFCGGS IISPRWVLTR AHCTTN---- ------TDPA AYTIRAGSTD
A. gam     ---RHNCGGS VLSSKWVLTA AHCTAG---- ------ASTS SLTVRLGTSR
D. mel     ---SHSCGGS IYSANIIVTA AHCLQS---- ------VSAS VLQVRAGSTY
H. arm     SAYWQSCGGT ILNNRAILTA AHCTAG---- ------DANN RWRIRVGSTW
S. per     P--RLFCGGS LINDRYVLTA SHCVHN---- ------NRDQ ITVRLLQLDR
Bov Chy    H----FCGGS LINENWVVTA AHCGVT---- ------TSDV VVAGEFDQGS
s621.2     ---------- ---------- ---------- DDIIKARTRT AVLLGSHDRS
s621.4     ---------- ---------- ---------- --PERNEPLS VVLAEHKVSS
s621.17    ---------- ---------- ------SDRW TKERTDPSDV APVVGAHSTS
s622.3     ---------- ---------- ---------- ----IGTSRK LYVVAGVDRI

           151                                                200
A. aeg     RTNG-GIIVK V---KSVIPH PQYN-GDTYN YDFSLLELDE SIGFSRSIEA
A. gam     HASG-GTVVR V---ARVVQH PKYD-SSSID FDYSLLELED ELTFSDAVQP
D. mel     WSSG-GVVAK V---SSFKNH EGYN-ANTMV NDIAVIRLSS SLSFSSSIKA
H. arm     ANSG-GVVHN L---AANIIH PSYN-SRTMD NDIAVLRSAT TFSFNNNVRA
S. per     SSRDPGITRQ V---SKVIMH PQYD-PVHIT NDVALLRLDT PVPFNDKIRP
Bov Chy    SSEKIQKLK- I---AKVFKN SKYN-SLTIN NDITLLKLST AASFSQTVSA
s621.2     RPSSTAVTIN V---ERINQH EKYN-ANTIA NDISILTLAS SINFNKVIGP
s621.4     KTESKTTIID V---TRMITH EQYDLKKNTE NDVALLVLAS KIPFGRTIGP
s621.17    QDSSHGSTQK LEVREIIIAD SWFDAKGSLK GDIAIIVLKE PMKYSKWVSP
s622.3     DTGVLNPVV- -----VAVVH KDYD-NTTNE NDLAVLHVSR DLNLDNKTRK

           201                                                250
A. aeg     IALPDASETV ADGAMCTVSG WGDTKNV--F EM-NTLLRAV NVPSYNQAEC
A. gam     VGLPKQDETV KDGTMTTVSG WGNTQSA--A ES-NAVLRAA NVPTVNQKEC
D. mel     ISL--ATYNP ANGASAAVSG WGTQSSG--S SSIPSQLQYV NVNIVSQSQC
H. arm     ASIAGANYNL ADNQAVWAAG WGTT-SA--G GSSSEQLRHV ELRSINQNTC
S. per     VCLPNKNHNF -DNKDAIVAG WGLIK-E--G GVTSNYLQEV TVPIITNQQC
Bov Chy    VCLPSASDDF AAGTTCVTTG WGLTRYT--N ANTPDRLQQA SLPLLSNTNC
s621.2     VCLPLPGLDV S-GQTVRVLG WGAERFQ--G AM-TMRPKKL DTTAVSPAQC
s621.4     ACFPKANLNI V-GQKVRVIG WGALASG--G RQ-PDILQKV DLDVQPTSAC
s621.17    VCLTPTQPDV V-DKYIRMMG WGAT--G--D GY-PDILREA NALVISPKDC
s622.3     -ALPIPTKVP LDGTLCRVSG WGSTVFDPVN PQPSRILLMT DVPLYNLGKC
```

```
                                                  Tryp3
          251                                                          300
A. aeg    AAALVNVVP- VTEQMICAGY -AAGGKDSCQ GDSGGPLVSG D----KLVGV
A. gam    NKAYSDFGG- VTDRMLCAGY -QQGGKDACQ GDSGGPLVAD G----KLVGV
D. mel    ASSTYGYGSQ IRNTMICA-- -AASGKDACQ GDSGGPLVSG G----VLVGV
H. arm    RNNYATRGIA ITANMLCSGW -PNGGRDQCQ GDSGGPLYHN G----IVVGV
S. per    RNTR--YKNK IFDVMLCAGL VKQGGKDACQ GDSGGPLIV- NEGRYKLAGV
Bov Chy   KKYW---GTK IKDAMICAG- --ASGVSSCM GDSGGPLVCK KNGAWTLVGI
s621.2    AAIWRGLVSA TNPTQVCTLS KKETA----- ---------- ----------
s621.4    SRVYKGITEG ----QLCTYT LRKDA----- ---------- ----------
s621.17   NND------- ---------- ---------- ---------- ----------
s622.3    R-----LELD VPIGQICAGY EDGGHDS--- ---------- ----------

          Tryp4
      301                                                  338
A. aeg    VSWGKGCALP NLPGVYARVS TVRQWIREVS EV------
A. gam    VSWGYGCAQA GYPGVYSRVA VVRDWVRENS GV------
D. mel    VSWGYGCAYS NYPGVYADVA VLRSWVVSTA NSI-----
H. arm    CSFGIGCAQA QFPGVNARVS RYTSWISSNA --------
S. per    VSFGFGCAQA NAPGVYARVS KFLDWIHNNS RDGCYCSG
Bov Chy   VSWGSSTCST STPGVYARVT ALVNWVQQTL AAN-----
s621.2    ---------- ---------- ---------- --------
s621.4    ---------- ---------- ---------- --------
s621.17   ---------- ---------- ---------- --------
s622.3    ---------- ---------- ---------- --------
```

Figure 4.14: Alignment of the predicted amino acid sequences of the serine proteinase PCR amplicons from the green mirid salivary gland mRNA (s621.2, s621.4 and s621.17) and midgut mRNA (s622.3), with *Aedes aegypti* trypsin 3A1 (*A. aeg*), *Anopheles gambiae* serine proteinase (*A. gam*), *Drosophila melanogaster* alpha trypsin (*D. mel*), *Helicoverpa armigera* trypsin (*H. arm*), *Sarcophaga peregrina* serine proteinase (*S. per*), and bovine chymotrypsin (Bov Chy). Primer sequences are not included in the amino acid translation of the mirid amplicons. Numbering is according to the bovine chymotrypsin system. Catalytic triad residues are boxed, and conserved cysteine residues are in bold. **Tryp X** indicates degenerate primers, and dashes represent gaps introduced to maximise the alignment.

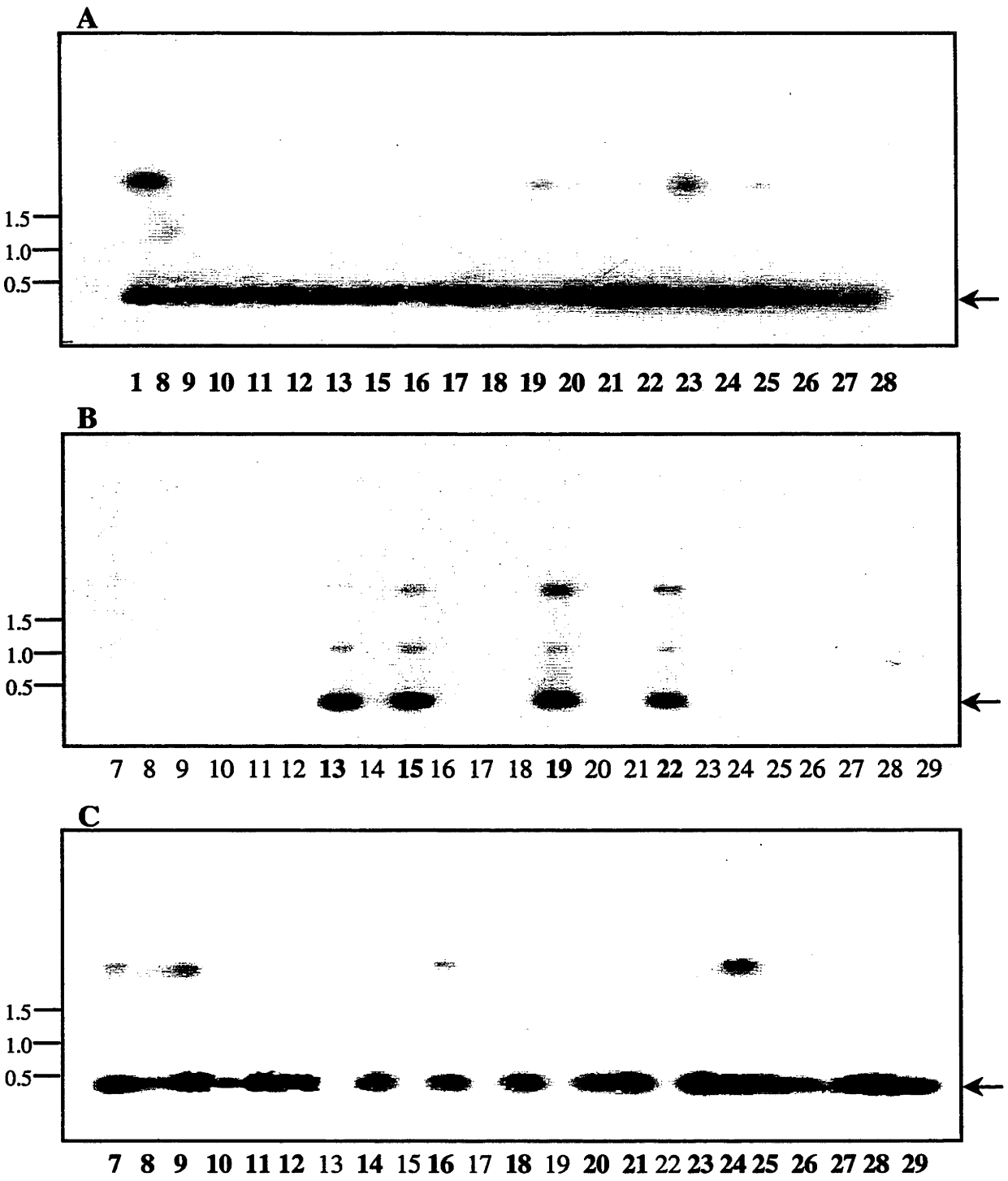

Fig 4.15: Southern blots of green mirid serine proteinase clones; **A**: midgut clones probed with PCR amplicon s622.3, **B**: salivary gland clones probed with PCR amplicon s621.2, and **C**: salivary gland clones probed with PCR amplicon s621.4. Numbers underneath represent clone numbers, and those in bold hybridised to the probe. Numbers on the left show approximate size in kb, and arrows indicate proteinase amplicons.

Table 4.4: Most similar nucleotide (nt) sequences to mirid serine proteinase PCR amplicons, determined by FastA searching of non redundant nucleotide databases.

Clone	Homologous sequence	% identity
s622.3	*Gadus morhua* (cod) trypsinogen gene	58.1% over 241 nt
	Pacifastacus leniusculus (crayfish) masquerade-like gene	58.7% over 208 nt
	Rattus norvegicus (rat) pancreatic trypsinogen gene	73.1% over 67 nt
s621.2	*D. melanogaster* (fruitfly) alpha trypsin gene	55.0% over 380 nt
	Culex pipiens quinquefasciatus (southern house mosquito) serine proteinase	57.7% over 234 nt
	Cochliobolus carbonum (fungus) trypsin-like proteinae	55.4% over 390 nt
s621.4	*Haliotis rufescens* (abalone) chymotrypsin-like gene	58.9% over 248 nt
	Lumbricus rubellus (earthworm)lumbrokinase	57.5% over 285 nt
	Helicoverpa armigera (cotton bollworm) serine proteinase	55.8% over 190 nt
s621.17	*Caenorhabditis elegans* (nematode) cosmid C45G9	69.9% over 73 nt
	Schizosaccharomyces pombe (fission yeast) cosmid c8C9	61.0% over 141 nt
	Rickettsia prowazekii (bacteria) genome	69.5% over 59 nt
CdSp1	*H. armigera* chymotrypsin-like proteinase	54.8% over 756 nt
	L. rubellus lumbrokinase	53.0% over 768 nt
	Manduca sexta (tobacco hornworm) hemocyte proteinase 1	56.1% over 360 nt

Table 4.5: Most similar sequences to the predicted amino acid (aa) sequences of mirid serine proteinase PCR amplicons, determined by FastA searching of non redundant protein databases.

Clone	Homologous sequence	% identity
s622.3	*Drosophila erecta* (fruitfly) trypsin zeta precursor	39.4% over 160 aa
	Culex pipiens quinquefasciatus trypsin precursor	35.7% over 154 aa
	Aedes aegypti (yellow fever mosquito) trypsin 3A1 precursor	35.9% over 156 aa
s621.2	*Homo sapiens* (human) chymotrypsin	32.5% over 157 aa
	Anopheles gambiae (African malaria mosquito) serine proteinase	30.5% over 154 aa
	Lucilia cuprina (Australian sheep blowfly) serine proteinase	30.1% over 153 aa
s621.4	*Sarcophaga peregrina* (fleshfly) proteinase	35.7% over 154 aa
	A. gambiae serine proteinase	34.4% over 154 aa
	Mus musculus (mouse) trypsinogen	33.3% over 153 aa
s621.17	*Tachypleus tridentatus* (horseshoe crab) limulus clotting factor C	30.6% over 134 aa
	Sus scrofa (pig) enteropeptidase	30.8% over 130 aa
	Astacus fluviatilis (broad-fingered crayfish) trypsin	29.2% over 130 aa
CdSp1	*G. morhua* chymotrypsinogen A	35.4% over 271 aa
	Canis familiaris (dog) chymotrypsinogen	34.1% over 264 aa
	A. gambiae serine proteinase	33.3% over 261 aa

Table 4.6: Sequence similarity (%) between mirid serine proteinase PCR amplicons.

	Nucleotide Identity	Amino Acid Identity	Amino Acid Similarity
s621.2/s621.4	51.9	38.8	54.4
s621.2/s621.17	40.1	29.6	50.4
s621.4/s621.17	47.3	26.7	49.2
s622.3/s621.2	41.6	28.3	44.8
s622.3/s621.4	47.0	31.7	49.3
s622.3/s622.17	48.2	16.7	36.0

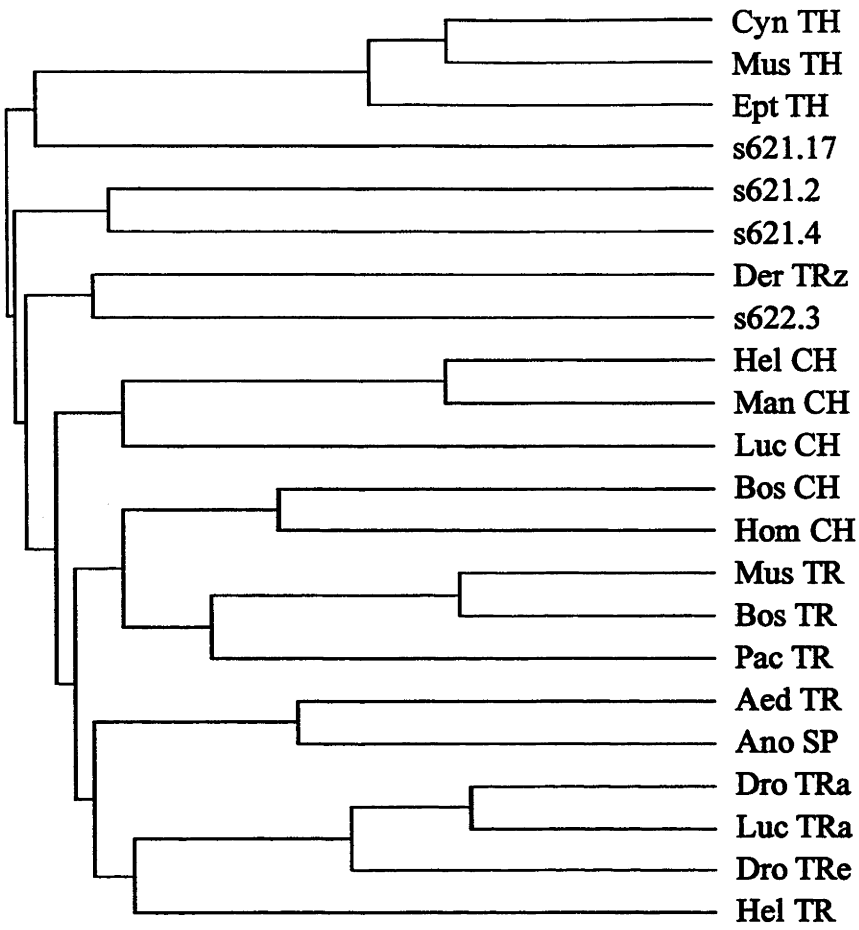

Figure 4.16: Mirid serine proteinase protein similarity tree, constructed with the Pileup function of the GCG software package. The following sequences, with accession numbers in brackets, were included in the analysis.

Cyn TH = *Cynops pyrogastor* (fire-bellied newt) thrombin (F42696); Mus TH = *Mus musculus* (mouse) thrombin (P19221); Ept TH = *Eptatretus stouti* (hagfish) thrombin (Q90504); s621.17, s621.2, s621.4 and s622.3 = *Creontiades dilutus* serine proteinase amplicons; Der TRz = *Drosophila erecta* (fruitfly) zeta trypsin (P54630); Hel CH = *Helicoverpa armigera* (cotton bollworm) chymotrypsin (O18445); Man CH = *Manduca sexta* (tobacco hornworm) chymotrypsin (L34168); Luc CH = *Lucilia cuprina* (Australian sheep blowfly) chymotrypsin (Q25227); Bos CH = *Bos taurus* (bovine) chymotrypsin (P00766); Hom CH = *Homo sapiens* (human) chymotrypsin (P40313); Mus TR = *M. musculus* trypsin (G2358087); Bos TR = *B. taurus* trypsin (P00760); Pac TR = *Pacifastacus leniusculus* (signal crayfish) trypsin (O46151); Aed TR = *Aedes aegypti* (yellow fever mosquito) trypsin (P29786); Ano SP = *Anopheles gambiae* (African malaria mosquito) serine proteinase (Q17039); Dro TRa = *Drosophila melanogaster* (fruitfly) alpha trypsin (P04814); Luc TRa = *L. cuprina* alpha trypsin (P35044); Dro TRe = *D. melanogaster* epsilon trypsin (P35005); Hel TR = *H. armigera* trypsin (O18447)

4.3.5 Cloning and Characterisation of a Salivary Serine Proteinase Gene

4.3.5.1 Cloning

An oligo dT primed cDNA library was made from whole adult mirid RNA. The library was screened at high stringency with the PCR amplicon of the salivary gland serine proteinase, s621.2. This gene was chosen for two reasons. Firstly, both the morphology and biochemistry of the green mirid imply the salivary glands play an important role in digestion, hence a salivary gland clone was chosen. Secondly, the biochemical data presented in Chapter 3 suggests chymotrypsin-like proteinase activity is predominant in the salivary glands, and s621.2 shows some homology to chymotrypsin genes in the FastA search results. Two positive clones were isolated and purified from the library. Phage DNA was isolated from both, and restriction enzyme digestion showed the first clone to have an insert approximately 210 bp in length. The second clone had an insert approximately 1 kb in length, and this clone, designated *Creontiades dilutus* Serine proteinase 1 (*CdSp1*), was characterised further.

4.3.5.2 Sequence Analysis of CdSp1

The entire clone of *CdSp1* was sequenced in both directions using specific internal primers and is a full length cDNA clone of a serine proteinase (Fig. 4.17). *CdSp1* corresponds exactly to the PCR amplicon s621.2 used for its isolation. Putative translation and termination codons are located at bases 19 and 898 respectively. *CdSp1* extends 18 nucleotides 5' of the predicted translation start site. The putative signal peptide cleavage site is located at amino acid 17 (nucleotide 67). The putative activation peptide cleavage site is located at amino acid 45 (nucleotide 151). The predicted protein sequence is 293 amino acids in length, and is shown aligned with serine proteinase genes with strong homology to *CdSp1* in Fig. 4.18.

The entire nucleotide sequence of *CdSp1* was used to search non redundant nucleotide databases, and showed strong homology to *Helicoverpa armigera* chymotrypsin-like serine proteinase (Table 4.4). The predicted amino acid sequence from the open reading frame was used to search non-redundant protein databases, and showed amino acid homology to chymotrypsin-like proteinases from cod and dog

(Table 4.5). All evidence suggests that *CdSp1* represents a chymotrypsin-like gene of the green mirid.

4.3.5.3 Expression of CdSp1

To determine the size of the RNA transcript, mRNA from total adult mirids was Northern blotted and probed with *CdSp1* labelled DNA (Fig. 4.19). A single transcript approximately 950 nucleotides in length hybridised to the probe.

Mirid size and scarcity meant it was not feasible to use Northern blot analysis to localise expression of *CdSp1* within the mirid. Instead, a DIG-labelled *CdSp1* RNA probe was used to localise expression in whole preparations of the salivary glands and midgut of the green mirid (Fig. 4.20). In addition to the antisense probe, a sense-strand probe was used to provide a negative control. As expected, the sense probe showed no significant hybridisation to RNA in either the salivary gland complex or the midgut of the green mirid. The antisense probe however showed strong hybridisation to the posterior lobe of the principal salivary gland. The antisense probe did not hybridise to the anterior lobe of the principal salivary gland, the accessory gland or the midgut.

```
  1  TTCGGTGTCCCATTCACGATGATGAAGTGGTTTATATTGGTTGCGGTGGTCGCTCTCGTC
  1                     M  M  K  W  F  I  L  V  A  V  V  A  L  V

 61  CAAGCCGACTCATCTGAGCACGGCGTTTCCCCAGGATCCAAAGGAACTACCTGCGCCTGC
 15  Q  A  D  S  S  E  H  G  V  S  P  G  S  K  G  T  T  C  A  C
  signal peptide cleavage site

121  GGGTGGGCCAACAGAAGTGGTGGATCCAGAATCGTGGGAGGAACCTACTACAAGGCCAAT
 35  G  W  A  N  R  S  G  G  S  R  I  V  G  G  T  Y  Y  K  A  N
                                  activation peptide cleavage site

181  GAATACCCATTCATTGTTGGTATTGCCACCGTCGGGGCCAGAGGATACGCGCCCTTCTGC
 55  E  Y  P  F  I  V  G  I  A  T  V  G  A  R  G  Y  A  P  F  C

241  GGTGGTTCCATCATCACTGCCAACCACGTCATCACTGCTGCTCACTGCACTGATGACATC
 75  G  G  S  I  I  T  A  N  H  V  I  T  A  A  H  C  T  D  D  I

301  ATCAAGGCCAGAACCAGGACCGCTGTTCTTTTGGGATCTCACGATCGCTCACGTCCCAGC
 95  I  K  A  R  T  R  T  A  V  L  L  G  S  H  D  R  S  R  P  S

361  TCTACTGCTGTTACCATCAACGTAGAAAGGATCAACCAGCACGAAAAATACAACGCCAAC
115  S  T  A  V  T  I  N  V  E  R  I  N  Q  H  E  K  Y  N  A  N

421  ACCATCGCCAACGACATTTCCATCCTCACTCTCGCCAGTTCCATCAACTTCAACAAGCTC
135  T  I  A  N  D  I  S  I  L  T  L  A  S  S  I  N  F  N  K  L

481  ATCGGACCAGTTTGCTTGCCACTTCCAGGTCTTGACGTTAGCGGACAGACTGTCAGAGTT
155  I  G  P  V  C  L  P  L  P  G  L  D  V  S  G  Q  T  V  R  V

541  CTTGGTTGGGGAGCTGAGAGGTTCCAGGGTGCCATGACTATGAGGCCCAAGAAGCTGGAC
175  L  G  W  G  A  E  R  F  Q  G  A  M  T  M  R  P  K  K  L  D

601  ACTACCGCAGTCTCTCCTGCTCAATGTGCTGCTATCTGGCGTGGATTGGTGTCAGCCACC
195  T  T  A  V  S  P  A  Q  C  A  A  I  W  R  G  L  V  S  A  T

661  AACCCAACCCAAGTGTGCACCCTTTCAAAGAAAGAGACCGCCTGCCAGGGAGATTCCGGT
215  N  P  T  Q  V  C  T  L  S  K  K  E  T  A  C  Q  G  D  S  G

721  GGCCCAGTCGTATGGCGTGACCCTCAGACCAACAGGTACACCCTCATTGGTCTTGTTTCC
235  G  P  V  V  W  R  D  P  Q  T  N  R  Y  T  L  I  G  L  V  S

781  TTCGGTGCCGCTTGTACTGATGAGAAGCCAACCGTCAACACCAGAGTCGCTGCTTACCTT
255  F  G  A  A  C  T  D  E  K  P  T  V  N  T  R  V  A  A  Y  L

841  CCATGGATCAAGCAACAAATCGCTGCTACCAAACCTGCCGGAGTTTGCACTAAGGCGTAA
275  P  W  I  K  Q  Q  I  A  A  T  K  P  A  G  V  C  T  K  A  *

901  TGGAAGACTGGGGATTCAAGACGTTTTATTAACTGAATTGAAGTTTACTTCACTCCAAAA

961  ATAAAACTACTTGTTACCAAAAAAA
```

Figure 4.17: Nucleotide and deduced amino acid sequences of *CdSp1* cDNA. The predicted signal peptide cleavage site and activation peptide cleavage site are shown. Possible translation initiation codon (ATG), translation termination codon (TAA), and polyadenylation signal (AATAAA), are underlined. ⟶ indicates primers used for sequencing.

```
                1                                                       7
H.arm Chy  MKLLAVTLLA FAAIVSARNI DLEDVIDLED ITAYDYHTKI GIPLAEKIRA
M.sex Chy  MKLAVVTLLA CASLAYGRSF NFEE--HLED ITAYGYLTKY GIPRAEEIRR
P.int Chy  --MASKILLC ILFVG----- -----VQSEV LTVHNYHMNI GVPRAINLMN
L.cup Chy  MKFLIVFVLA LAS------- ---------- ---------- ----------
A.aeg Chy  ---------- ---------- ---------- -------MAF KLTVAFLLVA
  Bov Chy  ---------- ---------- ---------- ---------- ---CGVPAIQ
D mel Try  ---------- ---------- ---------- -MLKIVILLS AVVCALGGTV
    CdSp1  ---------- --------MM KWFILVAVVA LVQADSSEHG VSPGSKGTTC

                8                                                      47
H.arm Chy  AEEEAERNPS RIVGGS-ISS LGAFPYQAGL LATFASG-QG ---VCGGSLL
M.sex Chy  MEEE.EIAQS RIVGGS-SSS VGQFPYQAGL VITLPRG-TA ---ACGGSLL
P.int Chy  SE-----LMT RIVGGSQVTT PTSFPFQAGI IATLTTGFTS ---ICGGTLL
L.cup Chy  ----ASAFEG RITNG-QDAV MGQFPYQVGL SLNLGNFKSA ---WCGGSLI
A.aeg Chy  SLALASSRAT HKIVGGDEAE AHEFPYQISL QWNFNDGQTE TMHFCGASVL
  Bov Chy  P--VLSGL-S -RIVNGEEAV PGSWPWQVSL Q------DKT GFHFCGGSLI
D mel Try  PEGLLPQLDG -RIVGGSATT ISSFPWQISL Q-------RS GSHSCGGSIY
    CdSp1  ACGWANRSGG SRIVGGTYYK ANEYPFIVGI A---TVGARG YAPFCGGSII

               48                                                      92
H.arm Chy  NNRRVLTAAH CWFDGRNQAR SFTVVLGS-V RLFSGGTR-- LNTASVVMHG
M.sex Chy  SNRRVLTAAH CWWDGQNQAS RFVVVLGS-N RLFSGGVR-- LETRDIVMHG
P.int Chy  SNTKVLTAAH CWWDGQSQAR LFTVVLGS-L TIFSGGTR-- IETSRIVVHP
L.cup Chy  GNEWVLTAAH C----TDGVK SVTVFLGA-T YRTEAEVKYT VKPNDILIHP
A.aeg Chy  NENFVLTAAH CKTAYSNTGF IEVVAAEH-D VAVAEGSEQR RLVAEFIVHE
  Bov Chy  NENWVVTAAH CGVTTSDV-- --VVAGEF-D QGSSSEKIQK LKIAKVFKNS
D mel Try  SANIIVTAAH CLQSVSAS-- --VLQVRA-G STYWSSGGVV AKVSSFKNHE
    CdSp1  TANHVITAAH CTDDIIKART RTAVLLGSHD RSRPSSTAVT INVERINQHE

               93                                                     140
H.arm Chy  SWNPNLIRND IAIINLPSNV ATSGNIAPIA LPSGNELNNN FNGATAVASG
M.sex Chy  SWNPNLVRND IAMIRLPSNV GFNNNINVIA LPSGSQLNLN FAGERAIASG
P.int Chy  NWNTNEITHD IAMVTIA-RV SFTNNIQSIP IPDLADINHN FAGASAVVSG
L.cup Chy  GWNNKTLKND ISLVKIP-ET AYTALIQPVE LPALASSYPS FAGDEVIASG
A.aeg Chy  DYQGGVSPDD IAVIRVDKPF ELNDKVKAVK LPKQLEQFD- -GDVT--LSG
  Bov Chy  KYNSLTINND ITLLKLSTAA SFSQTVSAVC LPSASDDFA- -AGTTCVTTG
D mel Try  GYNANTMVND IAVIRLSSSL SFSSSIKAIS L--ATYNPA- -NGASAAVSG
    CdSp1  KYNANTIAND ISILTLASSI NFNKLIGPVC LPLPG---LD VSGQTVRVLG

              141                                                     185
H.arm Chy  FGLANDGGS- --VDGNLRHV NLPVITNAVC TV---SFPGI IQSSNICTSG
M.sex Chy  FGRTRDGAN- --IDGSLNHV TLDVIANNVC SR---TFPLL IQSSNICTSG
P.int Chy  YGKTSDGQGS FPTTTSLHQT TVQVITNAVC QK---SFDIT LHGSHLCTNG
L.cup Chy  WGRISDSASG --VTNYLQWA RLEVISNAVC AR---TYGST ITSSNLCVKT
A.aeg Chy  WGSVSTTVFP -DYPDKLRKV VLPLVDYEQC DT-LWGNDSA LAKSNVCAGP
  Bov Chy  WGLTRYTN-A -NTPDRLQQA SLPLLSNTNC KKY---WGTK IKDAMICAGA
D mel Try  WGTQSSGS-S -SIPSQLQYV NVNIVSQSQC ASSTYGYGSQ IRNTMICAAA
    CdSp1  WGAERFQGAM TMRPKKLDTT A---VSPAQC AAIWRGLVSA TNPTQVCTLS

              186                                                     229
H.arm Chy  ANGR-STCQG DSGGPLV--V TSNNRRILIG VTSFGSARGC QV-GSPAAFA
M.sex Chy  ANGR-STCHG DSGGPLA--A TRNNRPLLIG VTSFGHRDGC QR-GHPAAFA
P.int Chy  QGGVGS-CDG DSGGPLT--T IRNNRRTVIG VVSFGLGDRC QS-GYPSVYT
L.cup Chy  PGGV-STCKG DSGGPLV--L ASSG--VQVG LTSFGSILGC EK-GFPAAFT
A.aeg Chy  IDGSKSACSA DSGGPLV--K QSGEEVIQVG VVSWGAVP-C GSPRRPTVFA
  Bov Chy  -SGV-SSCMG DSGGPLV--C KKNGAWTLVG IVSWGSST-C ST-STPGVYA
D mel Try  -SGK-DACQG DSGGPLV--S GG----VLVG VVSWGY--GC AYSNYPGVYA
    CdSp1  -KKE-TACQG DSGGPVVWRD PQTNRYTLIG LVSFGA--AC TD-EKPTVNT
                 *                          *          *
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          230                                 245
H.arm Chy  RVTSFISWIN NLL-------- ----
M.sex Chy  RVTSYDAWIR RNL-------- ----
P.int Chy  RVTAFLTWIQ ANL-------- ----
L.cup Chy  RVTSYLEWIN EHTGISY---- ----
A.aeg Chy  GVSHYVDWIE QQLRA------ ----
  Bov Chy  RVTALVNWVQ QTLAAN----- ----
D mel Try  DVAVLRSWVV STANSI----- ----
    CdSp1  RVAAYLPWIK QQIAATKPAGV CTKA
```

Figure 4.18: Alignment of the predicted amino acid sequence of *CdSp1* with the sequences of *Helicoverpa armigera* chymotrypsin (*H.arm* Chy), *Manduca sexta* chymotrypsin (*M.sex* Chy), *Plodia interpunctella* chymotrypsin (*P.int* Chy), *Lucilia cuprina* chymotrypsin (*L.cup* Chy), *Aedes aegypti* chymotrypsin (*A.aeg* Chy), bovine chymotrypsin (Bov Chy) and *Drosophila melanogaster* alpha trypsin (*D.mel* Try). Numbering is according to the bovine chymotrypsin system. ↑ indicates the activation peptide cleavage site. Catalytic triad residues (H57, D102, S195) are boxed. * below the alignment indicates binding pocket residues (D189, G216, G226 for trypsin-like proteinases). Conserved cysteine residues are in bold, and dashes represent gaps introduced to maximise the alignment.

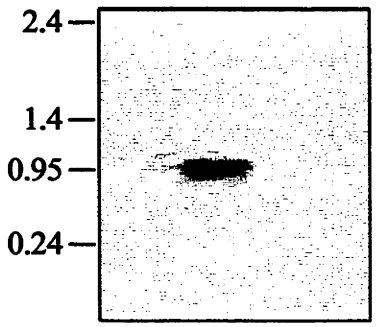
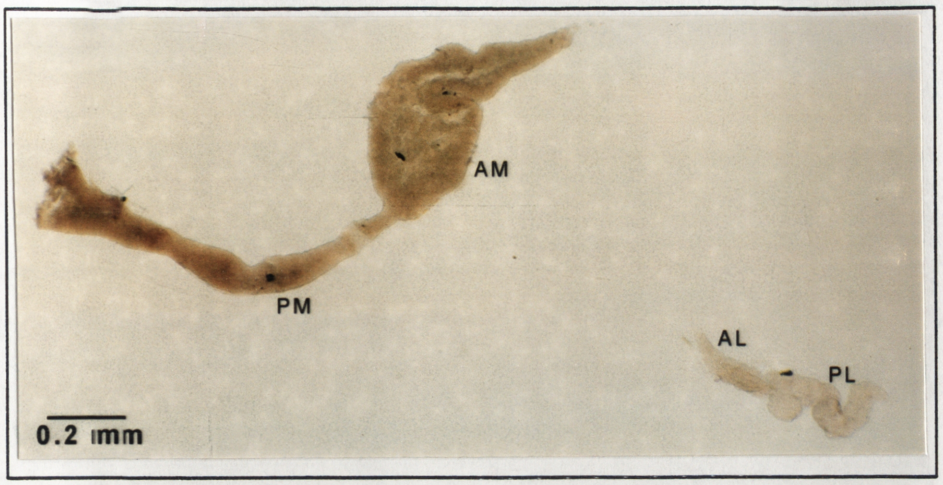


Figure 4.19: Northern blot of green mirid mRNA probed with *CdSp1*

A)



B)

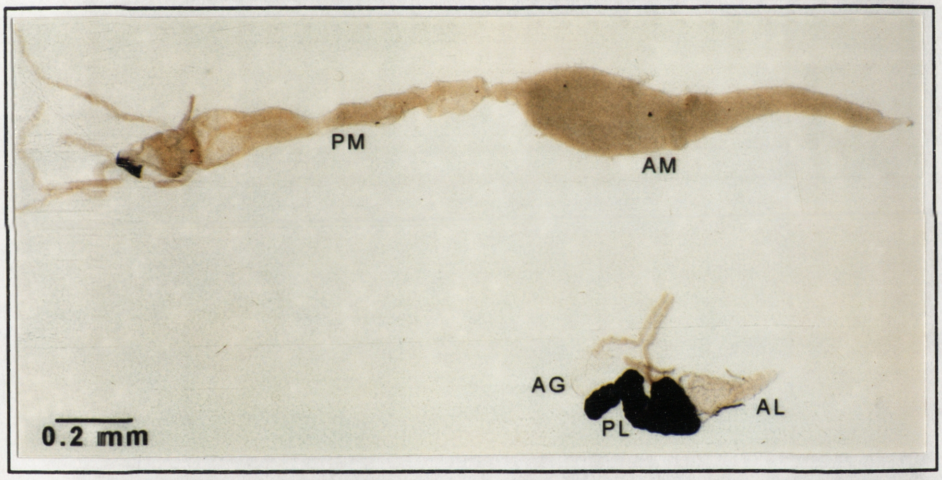


Figure 4.20: *In situ* hybridisation of *CdSp1*. **A)** Green mirid midgut and salivary gland probed with *CdSp1* in the sense orienation. **B)** Green mirid midgut and salivary gland probed with *CdSp1* in the antisense orienation.
PM, posterior midgut; AM, anterior midgut; AG, accessory salivary gland; AL, anterior lobe of principal salivary gland; PL, posterior lobe of principal salivary gland.

4.4 DISCUSSION

Green Mirid Cysteine Proteinases

No cysteine proteinase gene was identified from the salivary glands of the green mirid using RT-PCR. This suggests that no cysteine proteinase genes are expressed in the salivary glands, although the lack of amplification may have been due to non-optimal PCR conditions. The same RT-PCR conditions however did amplify cysteine proteinase gene fragments from the midgut of the green mirid. The second round of amplification in the half nested PCR produced a 500 bp fragment in addition to the 380 bp fragment expected. It was assumed that this larger amplicon was amplified by primer Cys 1, carried over in small amounts from the RT-PCR reaction, pairing with primer Cys 3, and it was not analysed any further. Sequence analysis of the PCR products revealed the presence of at least three different cysteine proteinases in the midgut of the green mirid. Southern blot analysis supported the presence of only three different midgut cysteine proteinse genes, with 16 of the 17 clones screened hybridising to one of the sequenced cysteine proteinase amplicons, and the last clone not representing a cysteine proteinase. In addition to hybridising to the proteinase amplicons, the probes hybridised to some larger fragments of DNA in the Southern blot analysis. These bands could represent either partial digests, or vector sequence which has hybridised to contaminating vector sequence in the probe.

The three cysteine proteinase amplicons have between 64% and 72% amino acid identity, which is only slightly higher than is typically seen among insect cysteine proteinases (62% to 68% is common). A protein similarity tree placed the three mirid cysteine proteinase amplicons in a large clade with vertebrate and invertebrate cysteine proteinases with cathepsin L homology (Fig. 4.11), separate to other cathepsin sequences. Within this cathepsin L clade, the mirid sequences group with the invertebrate sequences, but show the most similarity to each other, suggesting they share a recent common ancestor.

The presence of cysteine proteinase genes in the midgut of the green mirid supports the possible detection of cysteine proteinase activity in the midgut of the green

mirid in Chapter 3. It is also consistent with other studies which have detected cysteine proteinase activity in the guts of several heteropteran insects (Houseman, 1978; Houseman and Downe, 1981; Houseman *et al.*, 1984; Houseman *et al.*, 1985). While these other studies characterised the activity as cathepsin B like, the mirid cysteine proteinase genes and predicted protein sequences all show homology to cathepsin L type proteinases over the 380 bp cloned, and it is unlikely that obtaining the entire sequence of the mirid genes would significantly change the homologies. It is possible though that other cysteine proteinases, such as cathepsin B type proteinases, are expressed in the midgut of the green mirid but were either not amplified by the primers used, or were amplified but were not among the clones sequenced or screened. A third possibility is that other cysteine proteinases were among the clones screened by Southern hybridisation, and cross hybridised to the probes used. This seems unlikely since Qian *et al.* (1990) saw no cross hybridisation between rat cathepsins B, L, S or H at high stringency.

While it is possibile then that the mirid gut contains cathepsin L type proteinases, or even other types which were missed by the screening strategies employed, it is impossible to know from the sequence analysis alone what characteristics an enzyme will display, and hence to which group it actually belongs. Moreover, it seems rather restrictive to try and assign mirid gut proteinases to these specific categories, since by definition cathepsin proteinases are isolated from mammalian tissues and are more often housekeeping than digestive in function. There have simply been too few insect cysteine proteinases characterised and cloned to establish groups or types. From the results presented here it can only be asserted that there are at least three proteinases expressed in the midgut of the green mirid that belong to the cysteine mechanistic class of proteinases.

Midgut Serine Proteinases

Serine proteinase genes were amplified from the salivary glands and midgut of the green mirid. Six trypsin-like PCR amplicons were isolated from midgut mRNA which differed from each other by 1 to 12 nucleotides. Most of these differences could

be a result of PCR or sequencing errors. More notable was the presence of a four base pair insertion in three of the sequences (Fig. 4.12), causing a frameshift and introducing at least three stop codons. As well as stop codons, the predicted amino acid sequence of these amplicons lacks the active site serine at position 195, and so would be non-functional. This insertion is unlikely to be a sequencing error since the same insertion was detected in both the forward and reverse sequence reactions of three clones. It is possibly a PCR error, although this too would be unusual as PCR causes single base pair changes more often than insertion or deletion errors. The genes containing the insetion may not be expressed, and there is also a chance that contaminating genomic DNA was amplified, and that the sequences containing the insertion are not transcribed, although the mRNA purification and RT-PCR steps make this unlikely.

Accepting the insertion as real, two different serine proteinase amplicons were isolated from the midgut, one of which encodes a non-functional protein. The two amplicons could represent either two closely related genes, one of which is a pseudogene, or allelic variation in the population. Although insect trypsin genes are often found in clustered families, the different genes within a family are usually more divergent than observed here. For example, the two trypsin genes cloned from *Anopheles gambiae, Antryp1* and *Antryp2,* are only 70% identical at the nucleotide level (Müller *et al.*, 1993). This level of divergence however is not always observed. The three trypsin genes cloned from from a single larvae of *Manduca sexta* are at least 93% identical at the nucleotide level (Peterson *et al.*, 1994) which is more comparable to the 97% observed in the green mirid. It cannot be concluded from the data presented here whether the two midgut serine proteinase amplicons represent different genes or differeent alleles of a single gene. Southern blot analysis would help answer this question, and was attempted during this work without success as genomic DNA proved difficult to isolate intact from the green mirid.

Cladistic analysis groups the functional midgut serine proteinase in a large clade with vertebrate and invertebrate trypsin-like and chymotrypsin-like proteinases (Fig. 4.16). Within this clade, the midgut serine proteinase is most similar to the zeta trypsin from *Drosophila erecta.*

Salivary Gland Serine Proteinases

Three different serine proteinase genes were cloned from the salivary glands of the green mirid, which agrees with the findings of most other studies that serine proteinases are common in the salivary glands of the Heteroptera (Goodchild, 1952; Rastogi, 1962; Hori, 1970; Laurema *et al.*, 1985; Cohen, 1990). These three genes have between 26.7% and 38.8% amino acid identity, with s621.2 and s621.4 being the most similar. This level of homology is similar to that seen among insect serine proteinases in general (28% to 42% amino acid identity is common). A protein similarity tree placed s621.2 and s621.4 in a separate clade, grouped distantly with vertebrate and invertebrate trypsin-like and chymotrypsin-like proteinases including s622.3. This suggests that the s621.2 and s621.4 share a common ancestral gene and are quite divergent when compared to other serine proteinases. The same cladistic analysis placed s621.17 with vertebrate thrombin-like serine proteinases and separate from invertebrate sequences. This is interesting as it may indicate a function unrelated to digestion for s621.17. Thrombin is involved in coaggulation of blood in vertebrates. A thrombin-like proteinase which can clot plasma has been cloned from the salivary glands of mosquitoes (Bark *et al.*, 1996), where its action resembles a snake venom enzyme. In snakes, thrombin-like enzymes are toxic through their interference with the blood clotting process in bite victims, and while a possible function in mosquitoes was not put forward, it could well be playing a similar role in this hematophagous insect. In the green mirid which does not feed on blood it is more difficult to assign a role for a clotting enzyme. s621.17 may be present as a remnant from a carnivorous ancestor, in which it functioned as a toxin like in snakes. Alternatively, a clotting enzyme could be envisaged as having a role in the formation of hemipteran salivary sheaths, and so be present in the green mirid from a phytophagous ancestor.

In summary, three cysteine proteinases have been isolated from the midgut of the green mirid, and none from the salivary glands, using RT-PCR screening of an equal scale. In addition, two almost identical serine proteinase genes, one of which

does not encode a functional protein, have been identified from the midgut. Again in a screen of the same scale, three different serine proteinase genes have been identified in the salivary glands of the green mirid. These results imply that the midgut is the site of the majority of cysteine proteinase gene expression, while more serine proteinase genes are expressed in the salivary glands. The overall presence of multiple genes for a proteinase class is typical of insects, the general trend apparently being large families of enzymes (Davis *et al.,* 1985; Gatehouse *et al.,* 1997; Matsumoto *et al.,* 1997).

CdSp1

A cDNA, *CdSp1,* corresponding to one of the serine proteinase PCR amplicons from the salivary glands, was cloned and sequenced entirely and showed both nucleotide and amino acid homology to serine proteinase genes, particularly chymotrypsin-like genes. The presence of a signal peptide and activation peptide implies that *CdSp1* is a secreted enzyme active outside the cell. The amino acid sequence of *CdSp1* contains the three conserved residues of the catalytic triad present in all serine proteinases (His 57, Asp 102, Ser 195, numbered is according to bovine chymotrypsin). In addition the mirid sequence has Asp at position 194 (Fig. 4.15), a residue which is involved in stabilising the catalytic site. *CdSp1* also has six conserved cysteine residues which potentially allow the formation of three disulphide bridges. The binding pocket residues Asp 189, Gly 216, and Gly 226 are conserved in all trypsin-like proteinases and confer the specificity for basic side chains. The binding pocket residues for chymotrypsin-like proteinases are more variable, but are usually Ser 189, Gly 216, and Ala/Gly 226 (Jiang *et al.*, 1997). Gly 216, which lines the entry of the binding pocket, is conserved in *CdSp1*. The presence in *CdSp1* of Thr at position 189, which is at the bottom of the binding pocket, is more similar to the Ser of chymotrypsin sequences than to the negatively charged Asp 189 present in all trypsin sequences. However Thr at position 189 has not been seen before in insect chymotrypsins, and the effect of the slightly larger size of the side chain of Thr compared to that of Ser on the specificity of the enzyme is unknown. Thr is also found at position 226 in *CdSp1*, replacing the smaller Ala or Gly amino acids found in most

other known chymotrypsins. This substitution has also been observed in the *A. aegypti* chymotrypsin sequence (Jiang *et al.*, 1997), and a Ser is found in the same site in *P. interpunctella.* This binding pocket residue seems particularly variable, but the effect of these substitutions on substrate specificity is also unknown.

Northern analysis of *CdSp1* showed a single mRNA transcript approximately 950 nucleotides in length, in agreement with the size of the cDNA. *In situ* hybridisation experiments showed that *CdSp1* is expressed in the posterior lobe of the principal salivary gland, but not in the anterior lobe of the principal salivary gland, the accessory salivary gland, or the midgut. This agrees with the findings that the posterior lobe of the principal salivary gland is the site of digestive enzyme production in heteropteran insects (Miles, 1967). Furthermore, the strong hybridisation of *CdSp1* to apparently every cell in the posterior lobe indicates a high level of expression.

The presence of a chymotrypsin gene expressed in the salivary glands was predicted by the biochemical data presented in Chapter 3. However, it is not known whether the enzyme encoded by *CdSp1* is responsible for the chymotrypsin-like activity observed in the substrate and inhibitor assays. Purification and sequencing at least part of the enzyme from salivary glands, or expressing *CdSp1* and examining its biochemical properties *in vitro*, could contribute to answering this question.

CHAPTER 5: GENERAL DISCUSSION

The green mirid, *Creontiades dilutus,* is a significant Australian agricultural pest which, along with other sucking insects, is predicted to become even more economically important as management strategies for the control of chewing pests improve. Understanding the digestive physiology of these insects is essential for developing new control strategies, and yet there is a notable lack of studies on the digestive physiology of phytophagous Heteroptera. The aim of this thesis was to perform initial characterisation of the digestive system of the phytophagous heteropteran *C. dilutus.* This was first approached by examining the structure of the major digestive organs, the salivary glands and gut, of the green mirid. The second step was preliminary biochemical characterisation of digestive enzymes, focussing on proteinase activity. The biochemistry was then complemented by a molecular characterisation of proteinases expressed in the salivary glands and midgut of the green mirid. The following discussion will begin with a brief summary of the major results from this research.

5.1 SUMMARY OF RESULTS

The structure of the digestive system of the green mirid was described at the level of both gross morphology and cellular composition. The results did not differ markedly from similar studies of other cimicomorphan insects. The green mirid has an accessory salivary gland and a large, bi-lobed principal salivary gland, the two lobes of which differ in structure and in the appearance of the luminal contents. The midgut is a mildly acidic tube with a single cell type, although there is ultrastructural evidence that the cells of the anterior and posterior midgut perform different functions. Arguably the most notable feature of the midgut cell structure of many heteropteran insects is the functional replacement of a peritrophic membrane by extensive extracellular membrane layers, which were confirmed to be present in the green mirid also.

Amylase and proteinase activities were demonstrated in the salivary glands and midgut of the green mirid using general substrate assays. Both enzymes had higher

activity in the salivary glands than in the midgut, and neither showed any differences between adults and third instar nymphs or between fed and starved insects. Proteinase activity in the green mirid was characterised further, despite very low overall levels. Based on the pH optima and the inhibition of proteinase activity by serine proteinase inhibitors, it was concluded that serine proteinases predominate in salivary glands and secreted saliva of the green mirid. Specific substrate assays suggest the major serine proteinase active in the salivary glands is chymotrypsin-like in its specificity. RT-PCR using degenerate primers designed against cysteine proteinases did not succeed in amplifying any cysteine proteinases from the salivary glands, but the same technique using primers designed against serine proteinases amplified three different serine proteinase gene fragments from mirid salivary gland mRNA, supporting the predominance of this class of proteinase. One of the serine proteinase amplicons was used to isolate a full length cDNA clone, *CdSp1,* which corresponds to a putative chymotrypsin gene, and which was shown through *in situ* hybridisation to be expressed exclusively and at high levels in every cell of the posterior lobe of the principal salivary gland.

In contrast to the salivary glands, the acidic pH optimum of proteinases from midgut extracts implies that acidic proteinases predominate in the midgut of the green mirid. Inhibitor studies however suggest that both cysteine and serine proteinases are present. The presence of several bands of activity on zymograms supports the presence of a mixture of proteinases in the midgut of the green mirid, as does the molecular characterisation. Three different cysteine proteinase genes and one functional serine proteinase gene were amplified from the midgut mRNA using RT-PCR.

5.2 SALIVARY GLANDS OF THE GREEN MIRID

5.2.1 Salivary Gland Structure

The salivary glands are paired; each gland consists of a principal gland and an accessory gland. The accessory gland is smaller than the principal gland, and its function in the green mirid is unknown. In other Heteroptera it functions at least partly as a diuretic organ, producing a very dilute secretion (Miles, 1972), and it may be doing so here. In addition, HRP can travel intact from the haemolymph to the secreted saliva, and does so via the accessory gland, thus demonstrating an excretory function for the accessory gland (Miles and Sloviak, 1970). The absorption of horse radish peroxidase into the salivary glands of the green mirid after feeding implies an excretory function here too, perhaps also carried out by the accessory gland (see Chapter 2, section 2.3.3.2).

The structural differences between the two lobes of the principal salivary gland, the anterior lobe and the posterior lobe, imply they perform different functions. The posterior lobe of the principal salivary gland has previously been shown to be the site of digestive enzyme activity in heteropteran insects (Miles, 1967). Evidence of this function for the posterior lobe in the green mirid comes from the zymogram studies which demonstrated that the majority of proteinase activity is in the posterior lobe of the principal gland, compared with both the anterior lobe and the accessory gland. Also, the chymotrypsin gene cloned from the salivary glands of the green mirid, *CdSp1,* is expressed in the posterior lobe of the principal gland, and not in the anterior lobe, the accessory gland or the midgut. These pieces of evidence combine to suggest that salivary proteinase expression occurs predominantly in the posterior lobe of the principal gland of the green mirid.

5.2.2 Salivary Gland Enzymes

The most important enzymes of the salivary glands of the phytophagous bug *Lygus rugulipennis* were found to be polygalacturonase (pectinase), amylase and alkaline proteinase (Laurema *et al.*, 1985). The saliva of the green mirid also contains pectinase (Hori and Miles, 1993), as well as the amylase and proteinase activities demonstrated here. Pectinase activity has been suggested to have a role in the penetration of plant tissue for feeding (Strong and Kruitwagen, 1968), and possibly in preventing plant defences by intervening in plant wound response reactions (Miles, 1998). The α-amylase and proteinases in the saliva of the green mirid may aid penetration of the plant cells, and probably initiate external digestion of plant starches and proteins respectively, as they do in other mirids (Tingey and Pillemer, 1977). Evidence supporting this role for the proteinases in the green mirid includes the likely presence of zymogens in the secreted saliva. These proteinases become activated over time, and so are less likely to function primarily in the initial penetration of the plant.

Some early studies of heteropteran digestion found such low levels of enzyme activity in the salivary glands that pre-oral digestion was not considered feasible (Baptist, 1941). More recent studies indicate that salivary enzymes are an important component of overall digestion in most Heteroptera, with the notable exception of hematophagous insects (Terra *et al.*, 1988). For example, the salivary glands of predatory Heteroptera are the source of substantial proteinase and amylase activity (Cohen, 1990; Cohen, 1993). Pre-oral digestion in these insects allows the more efficient consumption of larger prey (Cohen, 1993). The extent of pre-oral digestion in phytophagous Heteroptera is less clear. Salivary enzymes are lacking from the seed-feeder *Dysdercus peruvianus,* (Pyrrhocoridae) and the contents of the seeds are dislodged by the action of the stylets with the copious saliva and digested internally (Silva and Terra, 1994). In contrast, the salivary glands of the phytophagous fourlined plant bug *Poecilocapsus lineatus* (Miridae) play an important role in digestion, as indicated by their large size and extensive rough endoplasmic reticulum, and by the immediate damage to the plant which occurs during feeding (Cohen and Wheeler, 1998). However, the only enzyme detected in the salivary glands of *P. lineatus* is

pectinase, and the only cells consumed by these insects are the pallisade cells of plants which are held together by a pectin matrix (Cohen and Wheeler, 1998). Such specific enzyme secretion has been seen before (Miles, 1987), but is not necessarily typical of phytophagous Heteroptera. Several different enzymes have been detected in the salivary glands of *Lygus rugulipennis* (Miridae) suggesting a more general role for pre-oral digestion in these bugs (Laurema *et al.*, 1985).

Several results suggest that pre-oral digestion is also major component of digestion in the green mirid. The salivary glands are relatively large, implying an important role in digestion. Both proteinase and amylase activities are higher in the salivary glands than in the midgut, again suggesting that salivary gland-originating enzymes are important. The molecular investigation of green mirid proteinases also indirectly supports a major role for pre-oral digestion, with three different serine proteinase genes being isolated from the salivary glands. At least one of these amplicons, *CdSp1,* has a signal peptide region suggestive of a secreted proteinase, and is expressed at high levels, indicating that this proteinase is likely to be a major component of the saliva. This appears to contradict the proteinase assay data which demonstrated only low levels of proteinase activity in the digestive system of the green mirid. More likely, the insensitivity of the biochemical assays is masking the true complexity of the situation.

5.3 MIDGUT OF THE GREEN MIRID

5.3.1 Midgut Structure

The gut of the green mirid is a simple tubular structure, dominated by the midgut region. The midgut is divided into three regions, the anterior sac-like region, and two posterior tubular sections divided by a constriction. The cells of the midgut are apparently all of one structural type, although ultrastructural analysis indicates that the anterior midgut is the site of the majority of fluid and ion regulation, as well as the major area for storage. The simplicity of the gut is indicated by the lack of a crop, gastric caeca, or a developed ileum. Also there is apparently no peritrophic membrane,

although extracellular membrane layers are present. The reduction in gut structural complexity has two major implications. Firstly, it is consistent with the ancestral Hemiptera being plant fluid feeders. This will be discussed in more detail later. Secondly, the gut structure observed for the green mirid, in being more similar to closely related species than to species with similar diets, demonstrates the feasibility of using digestive system structure in establishing taxonomic relationships.

5.3.2 Midgut Enzymes

Both α-amylase and proteinase activities were detected in the midgut of the green mirid. It is unknown whether the α-amylase detected in the midgut originated from the gut cells or from the salivary glands and was subsequently ingested with the food. However, the characteristics of the proteinase activity in the gut are different enough from those of the salivary proteinases to suggest that at least some of the proteinases are different.

The proteinases of the midgut appear to include both serine and cysteine proteinases. Having a variety of digestive proteinases has advantages for the insect. Besides digesting proteins for nutrition, proteinases may break down toxic defence proteins, such as lectins, produced by the plant (Peumans and van Damme, 1995). Proteinase inhibitors have also been implicated in plant defence systems (Ryan, 1990). There are plant proteinase inhibitor families specific to each of the four mechanistic classes of proteinases, although the majority are serine proteinase specific (Garcia-Olmedo *et al.*, 1987). Some of these proteinase inhibitors are ubiquitously expressed while others are induced by damage including insect attack. Insects seem to adapt to the presence of plant proteinase inhibitors by expressing inhibitor insensitive proteinases (Jongsma *et al.*, 1995). The presence of multiple proteinases, as is seen in the green mirid, is consistent with such adaptive responses. This is particularly pertinent for polyphagous insects, such as the green mirid, which may encounter a wide range of defence systems in their varied diet.

Houseman and Downe (1983) suggested that ingestion of salivary proteinases may contibute to protein digestion within the gut of some heteropteran insects. Cohen

(1993) has taken this further and proposed a model for digestive organisation in the predator *Zelus renardii* (Reduviidae) in which salivary gland endopeptidases begin protein digestion in the prey, then continue hydrolysis, possibly with midgut produced endopeptidases, in the anterior midgut. Exopeptidases in the anterior midgut and then the posterior midgut complete digestion of the proteins (Cohen, 1993). Digestion is organised quite differently in hematophagous Heteroptera where proteinase expression is limited to the posterior midgut (Houseman, 1978; Houseman and Downe, 1982a; Terra *et al.*, 1988). Cohen (1993) suggests that this difference is due to the different feeding habits of these groups of insects, since blood does not need to be liquefied before digestion compared to the solid nature of prey.

The likely importance of pre-oral digestion in the green mirid as discussed above (see section 5.2.2) is more similar to the predatory example, in which pre-oral digestion plays an important early role and is followed by sequential protein digestion in the gut (Cohen, 1993). In addition, the mirid salivary gland proteinases retain over half their maximum activity in slightly acidic environments such as the midgut. Salivary proteinases ingested with the food may therefore contribute to the continuation of protein digestion in the midgut of the green mirid, as is suggested for *Z. renardii* (Cohen, 1993). Finally, the anterior midgut of the green mirid seems likely to be active in digestive processes, which is again more similar to *Z. renardii* than to blood feeders where the anterior midgut functions primarily as a storage organ.

The ancestor of the Cimicomorpha was predaceous (Goodchild, 1963), consequently the predatory lifestyle is the most primitive in this group. Plant cells, like insect prey, provide a solid food source which requires partial digestion prior to ingestion through the stylets. Plant cell feeders have therefore retained a lacerate-and-flush style of feeding similar to their predatory ancestor (Miles, 1972), while blood feeders have become more specialised and diverged through, among other things, the loss of salivary enzyme activity. This may explain the similarity between the feeding style of the green mirid and *Z. renardii.* The similarity between feeding styles of phytophagous and predatory cimicomorphans may be why the green mirid, although not a good predator, is able to feed on other insects and insect eggs occasionally.

5.4 EVOLUTIONARY IMPLICATIONS

The fluid diet of the ancestral Hemiptera is presumed to have allowed a simplification of gut structure (Goodchild, 1966). The characteristics of this simplification include the absence of a crop, the absence of anterior midgut caeca, the loss of polymer hydrolase activity, and the absence of a peritrophic membrane (Terra, 1990). In all these ways, except perhaps the loss of polymer hydrolases, the gut of the green mirid resembles that of this proposed ancestor, adding to the body of evidence which suggests that these simplifications are common characteristics in the Hemiptera which arose early in the evolution of this order in a fluid feeding ancestor. Terra (1990) suggests that these modifications are adaptations to a phloem diet, which is low in complex molecules. However, as discussed in Chapter 1, it seems more likely that the diet of the ancestral Hemiptera consisted of plant cells rather than sap.

The proposed loss of enzyme activity in the ancestral hemipteran is suggested as the reason for the prevalence of cathepsin-like proteinases in the guts of the Heteroptera (Fig 5.1) (Houseman and Downe, 1983). Houseman and Downe (1983) suggest that the ancestral Hemiptera lost alkaline proteinase activity in the gut as an adaptation to the simplicity of a phloem diet. The ancestral Heteroptera, upon adopting a predaceous lifestyle and a more proteinaceous diet, utilised lysosomal catheptic proteinases to complete protein digestion in the gut. Cytochemical data on the luminal cathepsin B detected in *Rhodnius prolixus* are consistent with a lysosomal origin (Billingsley and Downe, 1988). The hypothesis described above is also supported by the widespread occurrence of cathepsins B and D in heteropteran families, including Reduviidae, Cimicadae, Phymatidae and Pentatomidae (Houseman, 1978; Houseman and Downe, 1982b; Houseman and Downe, 1983).

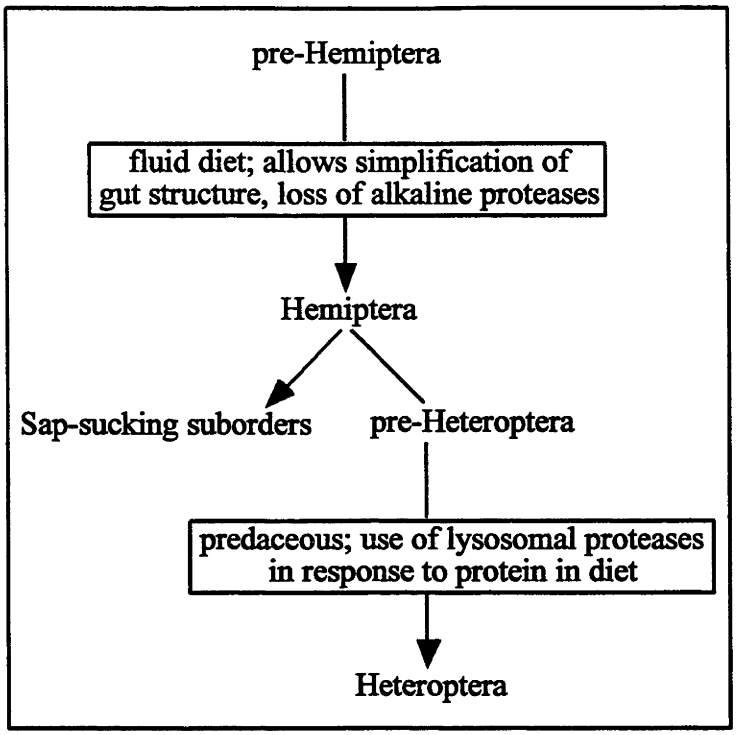


Figure 5.1: Possible evolutionary path leading to the modern Heteroptera (after Houseman and Downe, 1983).

There has been some debate as to the validity of this model, mostly surrounding the initial loss of proteinases in the ancestral Hemiptera (Terra *et al.*, 1988; Thie and Houseman, 1990). Assuming serine proteinase activity was lost, this may have occurred in one of two ways. A mutation in the regulatory region of the proteinase gene which reduced or abolished expression may have occurred. This loss in proteolytic activity would not have been particularly deleterious in an insect which was not ingesting many proteins, and so may have persisted. Alternatively, there may have been a mutation in the proteinase gene itself which resulted in the production of a non-functional, or at least much less active, enzyme. These latter two possibilities assume that either similar mutations occurred several times in the evolution of the Hemiptera, or else a single proteinase gene was responsible for serine proteinase activity in the ancestral Hemiptera. Considering that in many Heteroptera, including the green mirid, evidence suggests several proteinases are expressed in the digestive system, it seems

unlikely that a single mutation could have resulted in the loss of serine proteinase activity.

Regardless of how it may have occurred, the hypothesis that serine proteinases were lost in the ancestral Hemiptera is challenged by the presence of serine proteinases in the midgut of the green mirid. Terra *et al.* (1988) have also argued that the detection of alkaline proteinases in heteropteran insects disagrees with this hypothesis. However, of the three references quoted by Terra *et al.* to demonstrate heteropteran alkaline proteinases, only one (Khan, 1964) actually demonstrated the presence of alkaline proteinase activity in the digestive tract. The rest are studies on salivary proteinases. Salivary alkaline proteinases have been detected in several Heteroptera, including the green mirid in this thesis. Salivary proteinases have a different function to the gut enzymes, being involved in penetration of the food source and in pre-oral digestion of proteins. Throughout evolution, penetration of the food source always would have been a requirement, so that the alkaline salivary proteinases would not have been lost as is proposed for the gut proteinases. The presence of salivary alkaline proteinases therefore does not preclude the original loss of proteinase activity, and the subsequent use of lysosomal catheptic proteinases, in the gut as postulated.

There are however several other studies which have demonstrated the presence of alkaline proteinase activity in the midguts of heteropteran insects (Goodchild, 1952; Takanona and Hori, 1974; Cohen, 1993). Thie and Houseman (1990) argue that all alkaline proteinases detected in heteropteran insects are specialised salivary proteinases, so that the model of Houseman and Downe remains possible. This does not appear to be the case for the proteinases in the midgut of the green mirid. The green mirid midgut proteinases have a different pH optimum, different inhibitor profile, and different substrate preference, to the salivary proteinases. The banding pattern on zymogram gels is different in midgut and salivary gland extracts also. Thus the pool of enzymes in the midgut has a different composition to that of the salivary glands. The isolation of serine proteinase cDNA products from the midgut which are different to those identified from salivary glands strengthens the assertion that serine proteinases are expressed in the midgut of the green mirid, and that these are distinct from salivary

gland enzymes. The molecular characterisation demonstrating the expression of serine proteinase genes, together with the biochemical evidence for the presence of serine proteinase activiy, thus argue strongly for the presence of gut originating serine proteinases in the green mirid. While it is possible that these are not secreted extracellularly, their presence argues against an evolutionary model in which serine proteinases were lost from the midgut of the ancestral Hemiptera.

If serine proteinases were not lost from the ancestral Hemiptera, the question remains as to why acidic proteinases predominate in the guts of heteropteran insects. One possibility is that an ancestor to the Heteroptera fed on seeds rich in serine proteinase inhibitors, and adapted to this by using acidic proteinases not affected by the inhibitor for digestion (Terra *et al.*, 1988). A similar evolutionary event is proposed to have occurred in the ancestor of the seed feeding groups of the Coleoptera, in which acidic proteinases are also predominant (Terra and Cristofoletti, 1996). Indeed it has been suggested that seed feeding may have arisen on several occasions within the Pentatomomorpha, and that from these seed feeders arose the modern plant sap-sucking taxa, as well as the seed feeding taxa, of the Pentatomomorpha (Cobben, 1978). However it is on the Cimicomorpha, and not the Pentatomomorpha, that the majority of studies demonstrating the prevalence of acidic proteinases have focussed. No similar scenario of seed feeding ancestry has been proposed within the Cimicomorpha. Yet if the leaf litter dwelling heteropteran ancestor was omnivorous as suggested by Goodchild (1966), rather than exclusively predaceous as suggested by Cobben (1978), seeds may have formed part of its diet. The utilisation of lysosomal catheptic proteinases may have occurred here as an adaptation to seeds rich in trypsin inhibitors (Fig 5.2). This model involves the utilisation of lysosomal proteinases at the same point in evolution as the model proposed by Houseman and Downe, that is in the leaf litter-dwelling pre-Heteroptera. The difference is that in this scenario the use of acidic proteinases is an adaptation to seeds in the diet, rather than a means of replacing lost serine proteinases.

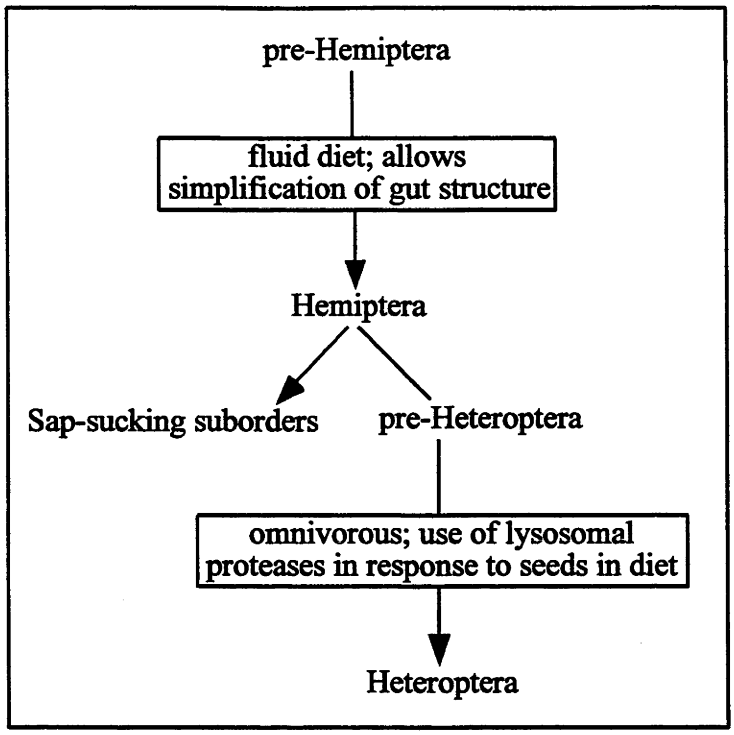


Figure 5.2: Alternative evolutionary path leading to the modern Heteroptera.

Some hematophagous insects, such as the mosquito *Aedes aegypti,* use trypsin-like enzymes even though blood is rich in trypsin inhibitors (Thie and Houseman, 1990). This appears to contradict the hypothesis that the presence of cysteine proteinases in heteropteran insects is an adaptation to seeds rich in serine proteinase inhibitors. Furthermore, seed feeding coleopteran insects which have predominantly cysteine proteinases also frequently have trypsin-like enzymes (Silva and Terra, 1994). The absence of serine proteinases from heteropteran midguts then would also disagree with the hypothesis of seed feeding ancestry (Silva and Terra, 1994). It seems though that serine proteinases are not actually absent from the midguts of all Heteroptera. Certainly the midgut of the green mirid appears to contain serine proteinases, and alkaline proteinase activity has been detected in the guts of several other Heteroptera (Goodchild, 1952; Takanona and Hori, 1974; Cohen, 1993).

Serine proteinases may be present in even more heteropteran insects than is apparent, but at such low levels that detection is difficult. Many of the studies which

have characterised acidic proteinases in heteropteran insects did not specifically look for serine proteinase activity. An experimental approach such as the one taken here, which combines biochemical techniques examining enzyme activity and sensitive molecular techniques including the use of degenerate PCR primers, is a potentially powerful way of examining digestion, particularly in small insect such as the green mirid.

In summary, an evolutionary model has been proposed by Houseman and Downe (1983) to explain the prevalence of acidic proteinases in the Heteroptera. According to this model, the phloem feeding hemipteran ancestor lost serine proteinases due to low amounts of protein in the diet. The ancestral Heteroptera then utilised lysosomal catheptic proteinases upon adapting to a carnivorous diet (Fig 5.1). This model is weakened by the fact that the ancestral Hemiptera were more likely plant cell feeders than phloem feeders, and by the presence of serine proteinases in the guts of some Heteroptera including the green mirid. An alternative model is proposed, extending from suggestions made by Terra *et al.* (1988), in which serine proteinase activity was not necessarily lost during evolution, and lysosomal proteinases became predominant in response to the presence of seeds in the diet of the heteropteran ancestor (Fig 5.2). This alternative model relies strongly on the suggestion by Goodchild that the heteropteran ancestor was a leaf-litter dwelling omnivore (Goodchild, 1966). This model is supported by the proposed presence of serine proteinases in the midgut of the green mirid which are different to those in the salivary glands, since their presence means it is unlikely that serine proteinases were lost in the ancestral Hemiptera.

5.5 FUTURE WORK

To approach the evolutionary issues described above, proteinases from a wide range of Heteroptera need to be examined. The heteropteran insects studied should include groups of each feeding type, that is plant cell feeders, seed feeders, sap-suckers, predators and blood suckers. A combined biochemical and molecular approach would be preferable, using proteinase inhibitors and substrates on gut extracts, and RT-PCR with primers designed against proteinase genes, and with gut mRNA as the template. Specifically relating to the evolutionary models described above, serine proteinases which originate in the gut should be sought. Serine proteinases may not dominate in the guts of Heteroptera, but will probably be quite common.

In terms of characterising the digestive proteinases of the green mirid at a purely basic level, many questions remain. The results obtained regarding inhibitor and substrate specificity are confused by the likely presence of multiple proteinases in the extracts, particularly from the midgut. The first step to a more complete characterisation of the proteinases active in the digestive system of the green mirid would be the purification of the midgut and salivary gland extracts, for example by chromatography. Once purified, a wide range of inhibitors and specific substrates could be used to determine the class and properties of individual proteinases. Ultimately, each proteinase identified could be sequenced.

Even after such characterisation, the function of individual proteinases, whether they are secreted digestive proteinases or intracellular housekeeping proteinases, may remain elusive. The origin of the proteinases may also be unknown. For example, proteinases purified from gut extracts may have originally been secreted from the salivary glands, and then been ingested with the food. Subcellular localisation of the proteinase of interest may contribute to solving this. Antibodies raised against the proteinase may allow subcellular localisation by immuno-cytochemical analysis. Alternatively, the cells of the gut and salivary glands could be fractionated, and the fractions analysed either by Western analysis, or by specific enzyme assays of the fractions. Localisation of the proteinase to the cells of the gut would confirm a gut origin. Further localisation of the proteinase to the Golgi apparatus, secretory vesicles,

or microvillar membranes, would imply it is secreted and therefore digestive in function. These methods have been employed before to investigate the distribution of enzymes in insect guts (Espinoza-Fuentes *et al.*, 1987; Ferreira *et al.*, 1988).

Completing the analysis of proteinases in the digestive system of the green mirid should involve continuing the molecular work begun here. Each proteinase amplicon identified by PCR could be used to obtain a full length clone of the gene. Obtaining the sequence of each of these clones would contribute to understanding the mixture of proteinases present in each tissue. Sequence analysis may also contribute to the understanding of the function of each gene. For example a signal sequence, as is present in *CdSp1,* implies that the enzyme is secreted from the cell and therefore probably has a digestive function. Localising the expression of the genes may also be suggestive of function. For example, specific expression in digestive tissues may suggest a digestive function. The use of Northern analysis on individual tissues from an organism as small as the green mirid is difficult, and RT-PCR may be a more feasible approach. Alternatively, *in situ* hybridisation, as used here for *CdSp1,* can provide a high degee of spatial resolution.

The sequences of the proteinase genes could be compared to the amino acid sequences of any proteinases purified from the green mirid, and in this way the characteristics of the protein encoded may be available. If the gene does not correspond to any isolated proteinase, it may be possible to express the functional proteinase encoded by the gene in insect cells using a baculovirus expression vector system. The proteinase could then be characterised biochemically.

5.6 CONCLUSION

The structure of the digestive system of the green mirid is consistent with a fluid feeding hemipteran ancestor in which simplification of the gut occurred in response to the simplicity of a partially digested fluid diet. The proteinase activity in the salivary glands of the green mirid is dominated by chymotrypsin-like activity, while the midgut of the green mirid appears to contain both serine and cysteine proteinases. The presence of serine proteinases in the midgut challenges an evolutionary model in which serine proteinases were lost in the ancestral Hemiptera. It seems that the digestive system of the green mirid comprises a complex mix of digestive proteinases. Further characterisation of proteinase activity in the green mirid would provide more information on the functional organisation of digestion in this bug and to some extent in phytophagous Heteroptera in general, which in turn may allow a sensible approach to controlling sucking pests of agricultural crops. In addition, characterisation of proteinases in the digestive systems of a wide range of heteropteran insects may contribute to understanding heteropteran evolution.

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APPENDIX 1: HRP FEEDING TRIAL, RAW DATA AND STATISTICAL TESTS

A1.1 HRP Feeding Trial: Raw Data

KEY to Terms and Abbreviations:

Rep: Experimental replicate

Sample: Std x = Horse radish peroxidase (HRP) standard

adult = adult green mirid

nymph = third instar nymph

Food: N/A = not applicable

control = mirids were fed on 7.5% sucrose overnight

HRP = mirids were fed on 2.5 mg/ml HRP in 7.5% sucrose overnight

Tissue: N/A = not applicable

sgc = salivary gland complex extract

gut = midgut extract

haem = haemolymph

Mal. tub. = Malpighian tubule extract

Absorbance: Absorbance at 405 nm, read 15 minutes after addition of the substrate

Amt. protein: amount of protein (μg) in the sample.

Dilution: Factor by which the sample was diluted prior to assaying

Rep.	Sample	Food	Tissue	Absorbance	Dilution
1	Std 1	N/A	N/A	0	1
1	Std 2	N/A	N/A	0.409	1
1	Std 3	N/A	N/A	0.69	1
1	Std 4	N/A	N/A	1.342	1
1	Std 5	N/A	N/A	1.615	1
1	Std 6	N/A	N/A	1.711	1
1	adult	control	sgc	0.133	1
1	adult	control	gut	0.254	1
1	adult	control	haem	0.073	1
1	adult	control	Mal. tub.	0.118	1
1	adult	HRP	sgc	0.643	50
1	adult	HRP	gut	2.326	2000
1	adult	HRP	haem	0.675	50

Rep.	Sample	Food	Tissue	Absorbance	Dilution
1	adult	HRP	Mal. tub.	0.18	50
1	nymph	control	sgc	0.084	1
1	nymph	control	gut	0.133	1
1	nymph	control	haem	0.08	1
1	nymph	control	Mal. tub.	0.242	1
1	nymph	HRP	sgc	1.481	200
1	nymph	HRP	gut	0.684	1000
1	nymph	HRP	haem	1.24	200
1	nymph	HRP	Mal. tub.	1.723	200
2	Std 1	N/A	N/A	0	1
2	Std 2	N/A	N/A	0.541	1
2	Std 3	N/A	N/A	0.845	1
2	Std 4	N/A	N/A	1.296	1
2	Std 5	N/A	N/A	1.464	1
2	Std 6	N/A	N/A	2.429	1
2	adult	control	sgc	1.223	10
2	adult	control	gut	0.855	20
2	adult	control	haem	0.23	1
2	adult	control	Mal. tub.	0.701	10
2	adult	HRP	sgc	0.952	200
2	adult	HRP	gut	1.318	2000
2	adult	HRP	haem	3.074	1
2	adult	HRP	Mal. tub.	1.118	500
2	nymph	control	sgc	0.677	1
2	nymph	control	gut	0.801	200
2	nymph	control	haem	1.129	1
2	nymph	control	Mal. tub.	1.275	1
2	nymph	HRP	sgc	0.302	50
2	nymph	HRP	gut	2.408	4000
2	nymph	HRP	haem	0.87	200
2	nymph	HRP	Mal. tub.	2.608	1
3	Std 1	N/A	N/A	0	1
3	Std 2	N/A	N/A	0.214	1
3	Std 3	N/A	N/A	0.513	1
3	Std 4	N/A	N/A	0.807	1
3	Std 5	N/A	N/A	0.946	1
3	Std 6	N/A	N/A	1.267	1
3	adult	control	sgc	0.619	1
3	adult	control	gut	0.541	1
3	adult	control	haem	0.09	1
3	adult	control	Mal. tub.	0.5	1
3	adult	HRP	sgc	1.677	1
3	adult	HRP	gut	1.099	5000
3	adult	HRP	haem	0.984	1
3	adult	HRP	Mal. tub.	0.307	10
3	nymph	control	sgc	0.815	1
3	nymph	control	gut	0.406	200
3	nymph	control	haem	0.1	1
3	nymph	control	Mal. tub.	0.217	1
3	nymph	HRP	sgc	0.501	1
3	nymph	HRP	gut	0.74	4000
3	nymph	HRP	haem	0.459	20
3	nymph	HRP	Mal. tub.	0.186	10

Rep.	Sample	Food	Tissue	Absorbance	Dilution
4	Std 1	N/A	N/A	0	1
4	Std 2	N/A	N/A	0.122	1
4	Std 3	N/A	N/A	0.218	1
4	Std 4	N/A	N/A	0.337	1
4	Std 5	N/A	N/A	0.406	1
4	Std 6	N/A	N/A	0.504	1
4	adult	control	sgc	0.223	1
4	adult	control	gut	0.512	1
4	adult	control	haem	0.265	1
4	adult	control	Mal. tub.	0.539	1
4	adult	HRP	sgc	0.368	50
4	adult	HRP	gut	0.673	20000
4	adult	HRP	haem	0.576	10
4	adult	HRP	Mal. tub.	0.211	50
4	nymph	control	sgc	0.328	1
4	nymph	control	gut	0.37	1
4	nymph	control	haem	0.109	1
4	nymph	control	Mal. tub.	0.145	1
4	nymph	HRP	sgc	0.242	10
4	nymph	HRP	gut	0.317	10000
4	nymph	HRP	haem	0.669	10
4	nymph	HRP	Mal. tub.	0.384	20
5	Std 1	N/A	N/A	0	1
5	Std 2	N/A	N/A	0.566	1
5	Std 3	N/A	N/A	1.207	1
5	Std 4	N/A	N/A	1.774	1
5	Std 5	N/A	N/A	2.054	1
5	Std 6	N/A	N/A	2.495	1
5	adult	control	sgc	1.573	1
5	adult	control	gut	0.753	100
5	adult	control	haem	0.276	1
5	adult	control	Mal. tub.	0.201	10
5	adult	HRP	sgc	0.457	100
5	adult	HRP	gut	0.424	3000
5	adult	HRP	haem	0.396	500
5	adult	HRP	Mal. tub.	0.37	500
5	nymph	control	sgc	0.566	1
5	nymph	control	gut	0.573	1
5	nymph	control	haem	0.117	1
5	nymph	control	Mal. tub.	0.124	1
5	nymph	HRP	sgc	1.566	1
5	nymph	HRP	gut	0.681	5000
5	nymph	HRP	haem	3.226	1
5	nymph	HRP	Mal. tub.	0.618	1
6	Std 1	N/A	N/A	0	1
6	Std 2	N/A	N/A	0.671	1
6	Std 3	N/A	N/A	1.222	1
6	Std 4	N/A	N/A	1.79	1
6	Std 5	N/A	N/A	2.109	1
6	Std 6	N/A	N/A	2.283	1
6	adult	control	sgc	0.196	1
6	adult	control	gut	0.262	5
6	adult	control	haem	0.332	1

Rep.	Sample	Food	Tissue	Absorbance	Dilution
6	adult	control	Mal. tub.	0.387	1
6	adult	HRP	sgc	0.726	20
6	adult	HRP	gut	0.639	3000
6	adult	HRP	haem	0.759	3000
6	adult	HRP	Mal. tub.	1.282	300
6	nymph	control	sgc	0.331	1
6	nymph	control	gut	0.426	1
6	nymph	control	haem	0.206	1
6	nymph	control	Mal. tub.	0.112	1
6	nymph	HRP	sgc	0.687	50
6	nymph	HRP	gut	0.675	3000
6	nymph	HRP	haem	0.254	50
6	nymph	HRP	Mal. tub.	1.057	300

A1.2 HRP Feeding Trial: Analysis of Variance (ANOVA)

Variate: ln (Dilution x HRP conc.)

Source of Variation	d.f.	s.s	m.s.	v.r	F pr.
date stratum	5	56.117	11.223	1.83	
date.mirid stratum					
sample	1	5.941	5.941	0.97	0.340
food	1	567.528	567.528	92.68	<0.001
sample.food	1	2.206	2.206	0.36	0.557
Residual	15	91.849	6.123	2.08	
date.mirid.units stratum					
tissue	3	223.738	74.579	25.29	<0.001
sample.tissue	3	3.134	1.045	0.35	0.786
food.tissue	3	55.790	18.597	6.31	<0.001
sample.food.tissue	3	0.185	0.062	0.02	0.996
Residual	60	176.959	2.949		
Total	95	1183.447			

APPENDIX 2: AMYLASE ASSAY, RAW DATA AND STATISTICAL TESTS

A2.1 Amylase Assay: Raw Data

KEY to Terms and Abbreviations:

Rep: Experimental replicate

Sample: adult = adult green mirid

nymph = third instar nymph

blank = water only

Feed: starved = fed water only overnight

fed = fed green beans and lettuce overnight

N/A: not applicable for blanks

Tissue: sgc = salivary gland complex extract

gut = midgut extract

N/A: not applicable for blanks

Amt. protein: amount of protein (µg) in the sample

Time: time (hr) of absorbance reading, from the start of the reaction

Absorbance: Absorbance of sample at 620nm

Rep	Sample	Feed	Tissue	Amt. protein	Time	Absorbance
1	adult	starved	sgc	2.06	0.00	0.013
					0.50	0.109
					1.58	0.278
					3.25	0.573
1	adult	starved	gut	5.05	0.00	0.010
					0.50	0.043
					1.58	0.091
					3.25	0.189
1	adult	fed	sgc	1.97	0.00	0.011
					0.50	0.048
					1.58	0.151
					3.25	0.256
1	adult	fed	gut	4.25	0.00	0.011
					0.50	0.038
					1.58	0.126
					3.25	0.260
1	nymph	starved	sgc	2.76	0.00	0.011
					0.50	0.040
					1.58	0.118
					3.25	0.268
1	nymph	starved	gut	4.68	0.00	0.014
					0.50	0.114
					1.58	0.278
					3.25	0.578

Rep	Sample	Feed	Tissue	Amt. protein	Time	Absorbance
1	nymph	fed	sgc	4.06	0.00	0.008
					0.50	0.037
					1.58	0.061
					3.25	0.121
1	nymph	fed	gut	5.60	0.00	0.011
					0.50	0.053
					1.58	0.135
					3.25	0.308
1	blank	N/A	N/A	0.00	0.00	0.011
					0.50	0.021
					1.58	0.030
					3.25	0.091
2	adult	starved	sgc	6.24	0.00	0.006
					1.00	0.234
					2.00	0.431
					3.25	0.775
2	adult	starved	gut	9.61	0.00	0.008
					1.00	0.092
					2.00	0.211
					3.25	0.347
2	adult	fed	sgc	11.90	0.00	0.006
					1.00	0.217
					2.00	0.493
					3.25	0.903
2	adult	fed	gut	14.80	0.00	0.007
					1.00	0.102
					2.00	0.215
					3.25	0.315
2	nymph	starved	sgc	8.33	0.00	0.005
					1.00	0.489
					2.00	1.049
					3.25	1.901
2	nymph	starved	gut	10.20	0.00	0.008
					1.00	0.141
					2.00	0.389
					3.25	0.583
2	nymph	fed	sgc	7.51	0.00	0.007
					1.00	0.391
					2.00	1.132
					3.25	1.856
2	nymph	fed	gut	9.15	0.00	0.006
					1.00	0.184
					2.00	0.455
					3.25	0.830
2	blank	N/A	N/A	0.00	0.00	0.005
					1.00	0.029
					2.00	0.053
					3.25	0.081
3	adult	starved	sgc	3.07	0.00	0.010
					1.25	0.018
					2.00	0.020
					3.00	0.021
3	adult	starved	gut	2.30	0.00	0.012
					1.25	0.290
					2.00	0.407
					3.00	0.677
3	adult	fed	sgc	4.23	0.00	0.012
					1.25	0.133
					2.00	0.218
					3.00	0.350

Rep	Sample	Feed	Tissue	Amt. protein	Time	Absorbance
3	adult	fed	gut	8.70	0.00	0.009
					1.25	0.086
					2.00	0.140
					3.00	0.196
3	nymph	starved	sgc	2.30	0.00	0.010
					1.25	0.271
					2.00	0.415
					3.00	0.690
3	nymph	starved	gut	4.23	0.00	0.011
					1.25	0.019
					2.00	0.031
					3.00	0.036
3	nymph	fed	sgc	1.74	0.00	0.012
					1.25	0.041
					2.00	0.065
					3.00	0.085
3	nymph	fed	gut	6.60	0.00	0.011
					1.25	0.065
					2.00	0.108
					3.00	0.159
3	blank	N/A	N/A	0.00	0.00	0.008
					1.25	0.029
					2.00	0.038
					3.00	0.050
4	adult	starved	sgc	6.67	0.00	0.000
					1.00	0.145
					2.00	0.352
					3.00	0.476
4	adult	starved	gut	14.85	0.00	0.001
					1.00	0.274
					2.00	0.512
					3.00	0.741
4	adult	fed	sgc	9.60	0.00	0.000
					1.00	0.613
					2.00	1.128
					3.00	1.862
4	adult	fed	gut	12.40	0.00	0.000
					1.00	0.101
					2.00	0.168
					3.00	0.245
4	nymph	starved	sgc	4.41	0.00	0.000
					1.00	0.151
					2.00	0.255
					3.00	0.398
4	nymph	starved	gut	5.79	0.00	0.000
					1.00	0.207
					2.00	0.318
					3.00	0.576
4	nymph	fed	sgc	11.85	0.00	0.000
					1.00	0.037
					2.00	0.074
					3.00	0.116
4	nymph	fed	gut	6.50	0.00	0.000
					1.00	0.039
					2.00	0.076
					3.00	0.127
4	blank	N/A	N/A	0.00	0.00	0.000
					1.00	0.006
					2.00	0.015
					3.00	0.011

Rep	Sample	Feed	Tissue	Amt. protein	Time	Absorbance
5	adult	starved	sgc	3.95	0.00	0.009
					1.00	0.413
					2.00	0.660
					3.50	1.170
5	adult	starved	gut	7.71	0.00	0.007
					1.00	0.040
					2.00	0.070
					3.50	0.158
5	adult	fed	sgc	4.23	0.00	0.009
					1.00	0.388
					2.00	0.658
					3.50	1.072
5	adult	fed	gut	8.59	0.00	0.013
					1.00	0.167
					2.00	0.334
					3.50	0.466
5	nymph	starved	sgc	5.79	0.00	0.008
					1.00	0.063
					2.00	0.115
					3.50	0.226
5	nymph	starved	gut	9.29	0.00	0.010
					1.00	0.151
					2.00	0.275
					3.50	0.424
5	nymph	fed	sgc	7.81	0.00	0.007
					1.00	0.152
					2.00	0.260
					3.50	0.415
5	nymph	fed	gut	9.98	0.00	0.006
					1.00	0.085
					2.00	0.188
					3.50	0.301
5	blank	N/A	N/A	0.00	0.00	0.010
					1.00	0.015
					2.00	0.025
					3.50	0.026
6	adult	starved	sgc	5.31	0.00	0.012
					1.00	0.311
					2.10	0.621
					3.00	0.820
6	adult	starved	gut	9.85	0.00	0.012
					1.00	0.054
					2.10	0.138
					3.00	0.173
6	adult	fed	sgc	7.01	0.00	0.013
					1.00	0.220
					2.10	0.394
					3.00	0.518
6	adult	fed	gut	9.61	0.00	0.013
					1.00	0.169
					2.10	0.293
					3.00	0.739
6	nymph	starved	sgc	5.08	0.00	0.010
					1.00	0.126
					2.10	0.203
					3.00	0.566
6	nymph	starved	gut	7.12	0.00	0.010
					1.00	0.033
					2.10	0.117
					3.00	0.120

Rep	Sample	Feed	Tissue	Amt. protein	Time	Absorbance
6	nymph	fed	sgc	5.59	0.00	0.012
					1.00	0.049
					2.10	0.100
					3.00	0.132
6	nymph	fed	gut	5.17	0.00	0.012
					1.00	0.072
					2.10	0.169
					3.00	0.273
6	blank	N/A	N/A	0.00	0.00	0.010
					1.00	0.034
					2.10	0.029
					3.00	0.066

A2.2 Amylase Assay: Secreted Saliva Raw Data

Rep	Sample	Time	Absorbance
1	saliva	0	0.009
		3	0.331
1	blank	0	0.007
		3	0.033
2	saliva	0	0.010
		3	0.114
2	blank	0	0.010
		3	0.015
3	saliva	0	0.010
		3	1.866
3	blank	0	0.010
		3	0.015
4	saliva	0	0.014
		3	0.363
4	blank	0	0.01
		3	0.052
5	saliva	0	0.012
		3	0.317
5	blank	0	0.01
		3	0.052

A2.3 Amylase Assay: Analysis of Variance (ANOVA)

A2.3.1 Variate: slope

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
date stratum	5	0.225	0.0449	2.94	
date.mirid.stratum					
sample	1	0.0102	0.0102	0.67	0.427
feed	1	0.000680	0.000680	0.04	0.836
sample.feed	1	0.0144	0.0144	0.95	0.346
Residual	15	0.229	0.0153	0.88	
date.mirid.part stratum					
tissue	1	0.905	0.905	5.23	0.033
sample.tissue	1	0.00619	0.00619	0.36	0.557
feed.tissue	1	0.000240	0.000240	0.01	0.907
sample.feed.tissue	1	0.0114	0.0114	0.66	0.426
Residual	20	0.346	0.0173		
Total	47	0.933			

A2.3.2 Variate: response (ln [slope/protein])

Source of Variation	d.f. (m.v.)	s.s.	m.s.	v.r.	F pr.
date stratum	5	3.037	0.607	1.18	
date.mirid.stratum					
sample	1	0.550	0.550	1.07	0.318
feed	1	0.671	0.671	1.30	0.272
sample.feed	1	1.114	1.114	2.16	0.162
Residual	15	7.726	0.515	0.39	
date.mirid.part stratum					
tissue	1	6.624	6.624	4.99	0.037
sample.tissue	1	0.496	0.496	0.37	0.548
feed.tissue	1	0.255	0.255	0.19	0.666
sample.feed.tissue	1	1.863	1.863	1.40	0.250
Residual	20	26.561	1.328		
Total	47	48.896			

APPENDIX 3: GENERAL PROTEASE ASSAY (AZOCASEIN), RAW DATA AND STATISTICAL TESTS

A3.1 General Proteinase Assay (Azocasein): Raw Data

KEY to Terms and Abbreviations:

Rep: Experimental replicate

Sample: blank = buffer (0.15 M Tris-Cl, pH 8.0) only
sgc = salivary gland complex extract
gut = midgut extract
conts = midgut luminal contents
saliva = secreted saliva
tryp = trypsin (positive control)

Sex: pooled = male and female samples mixed randomly
male = tissue extracted from male mirids only
female = tissue extracted from female mirids only
n/a = not applicable for the sample

Feed: fed = fed green beans and lettuce overnight
starved = fed water only overnight

Age: adult = adult green mirid
nymph = third instar nymph

Activators: EDTA/DTT = 3 mM EDTA + 1 mM DTT
EDTA/DTT(b) = 5 mM EDTA + 3 mM DTT

Inhibitors: AEBSF = 0.25 mg/ml AEBSF
antipain = 50 µg.ml antipain
antipain(b) = 100 µg/ml antipain
E-64 = 4.2 µg/ml E-64
E-64(b) = 16.7 µg/ml E-64
E-64(c) = 25 µg/ml E-64
E-64(d) = 50 µg/ml E-64
aprotinin = 2.0 µg/ml aprotinin
AEBSF/E-64 = 0.5 mg/ml AEBSF + 16.7 µg/ml E-64
AEBSF/E-64(b) = 0.5 mg/ml AEBSF + 50 µg/ml E-64

Time: time (hour) of absorbance reading, from the start of the reaction (time = 0)

Absorbance: absorbance of sample at 340 nm

Amt. protein: concentration of protein in the sample (mg/ml)

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
1	blank	n/a	fed	n/a			0.00	0.234	0.000
1	blank	n/a	fed	n/a			3.17	0.246	
1	blank	n/a	fed	n/a			11.17	0.270	
1	blank	n/a	fed	n/a			21.33	0.325	
1	sgc	pooled	fed	adult			0.00	0.246	N/D
1	sgc	pooled	fed	adult			3.17	0.269	
1	sgc	pooled	fed	adult			11.17	0.352	
1	sgc	pooled	fed	adult			21.33	0.501	
1	gut	pooled	fed	adult			0.00	0.254	N/D
1	gut	pooled	fed	adult			3.17	0.309	
1	gut	pooled	fed	adult			11.17	0.420	
1	gut	pooled	fed	adult			21.33	0.568	
1	sgc	pooled	fed	adult	EDTA/DTT		0.00	0.251	N/D
1	sgc	pooled	fed	adult	EDTA/DTT		3.17	0.258	
1	sgc	pooled	fed	adult	EDTA/DTT		11.17	0.301	
1	sgc	pooled	fed	adult	EDTA/DTT		21.33	0.374	
1	gut	pooled	fed	adult	EDTA/DTT		0.00	0.258	N/D
1	gut	pooled	fed	adult	EDTA/DTT		3.17	0.387	
1	gut	pooled	fed	adult	EDTA/DTT		11.17	0.541	
1	gut	pooled	fed	adult	EDTA/DTT		21.33	0.710	
1	tryp	n/a	fed	n/a			0.00	0.248	0.010
1	tryp	n/a	fed	n/a			3.17	0.604	
1	tryp	n/a	fed	n/a			11.17	1.027	
1	tryp	n/a	fed	n/a			21.33	1.615	
2	blank	n/a	fed	n/a			0.00	0.200	0.000
2	blank	n/a	fed	n/a			4.67	0.222	
2	blank	n/a	fed	n/a			10.58	0.268	
2	blank	n/a	fed	n/a			22.75	0.303	
2	sgc	female	fed	adult			0.00	0.283	N/D
2	sgc	female	fed	adult			4.67	0.401	
2	sgc	female	fed	adult			10.58	0.568	
2	sgc	female	fed	adult			22.75	0.911	
2	gut	female	fed	adult			0.00	0.288	N/D
2	gut	female	fed	adult			4.67	0.309	
2	gut	female	fed	adult			10.58	0.341	
2	gut	female	fed	adult			22.75	0.407	
2	tryp	n/a	fed	n/a			0.00	0.246	0.010
2	tryp	n/a	fed	n/a			4.67	0.654	
2	tryp	n/a	fed	n/a			10.58	1.042	
2	tryp	n/a	fed	n/a			22.75	2.061	
3	blank	n/a	fed	n/a			0.00	0.215	0.000
3	blank	n/a	fed	n/a			3.42	0.232	
3	blank	n/a	fed	n/a			7.22	0.246	
3	blank	n/a	fed	n/a			21.00	0.289	
3	sgc	pooled	fed	adult			0.00	0.204	N/D
3	sgc	pooled	fed	adult			3.42	0.240	
3	sgc	pooled	fed	adult			7.22	0.279	
3	sgc	pooled	fed	adult			21.00	0.432	
3	gut	pooled	fed	adult			0.00	0.218	N/D
3	gut	pooled	fed	adult			3.42	0.227	
3	gut	pooled	fed	adult			7.22	0.245	
3	gut	pooled	fed	adult			21.00	0.312	
3	sgc	pooled	fed	adult	EDTA/DTT		0.00	0.221	N/D
3	sgc	pooled	fed	adult	EDTA/DTT		3.42	0.229	
3	sgc	pooled	fed	adult	EDTA/DTT		7.22	0.252	
3	sgc	pooled	fed	adult	EDTA/DTT		21.00	0.379	
3	gut	pooled	fed	adult	EDTA/DTT		0.00	0.214	N/D

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
3	gut	pooled	fed	adult	EDTA/DTT		3.42	0.212	
3	gut	pooled	fed	adult	EDTA/DTT		7.22	0.223	
3	gut	pooled	fed	adult	EDTA/DTT		21.00	0.274	
3	sgc	pooled	fed	adult	EDTA/DTT(b)		0.00	0.214	N/D
3	sgc	pooled	fed	adult	EDTA/DTT(b)		3.42	0.221	
3	sgc	pooled	fed	adult	EDTA/DTT(b)		7.22	0.254	
3	sgc	pooled	fed	adult	EDTA/DTT(b)		21.00	0.336	
3	gut	pooled	fed	adult	EDTA/DTT(b)		0.00	0.217	N/D
3	gut	pooled	fed	adult	EDTA/DTT(b)		3.42	0.218	
3	gut	pooled	fed	adult	EDTA/DTT(b)		7.22	0.222	
3	gut	pooled	fed	adult	EDTA/DTT(b)		21.00	0.258	
3	tryp	n/a	fed	n/a			0.00	0.218	0.010
3	tryp	n/a	fed	n/a			3.42	0.962	
3	tryp	n/a	fed	n/a			7.22	1.317	
3	tryp	n/a	fed	n/a			21.00	2.142	
4	blank	n/a	fed	n/a			0.00	0.205	0.000
4	blank	n/a	fed	n/a			2.83	0.227	
4	blank	n/a	fed	n/a			16.92	0.262	
4	blank	n/a	fed	n/a			29.33	0.282	
4	sgc	pooled	fed	adult			0.00	0.202	N/D
4	sgc	pooled	fed	adult			2.83	0.230	
4	sgc	pooled	fed	adult			16.92	0.372	
4	sgc	pooled	fed	adult			29.33	0.404	
4	gut	pooled	fed	adult			0.00	0.210	N/D
4	gut	pooled	fed	adult			2.83	0.244	
4	gut	pooled	fed	adult			16.92	0.362	
4	gut	pooled	fed	adult			29.33	0.404	
4	sgc	pooled	fed	adult	EDTA/DTT		0.00	0.226	N/D
4	sgc	pooled	fed	adult	EDTA/DTT		2.83	0.236	
4	sgc	pooled	fed	adult	EDTA/DTT		16.92	0.337	
4	sgc	pooled	fed	adult	EDTA/DTT		29.33	0.364	
4	gut	pooled	fed	adult	EDTA/DTT		0.00	0.204	N/D
4	gut	pooled	fed	adult	EDTA/DTT		2.83	0.245	
4	gut	pooled	fed	adult	EDTA/DTT		16.92	0.327	
4	gut	pooled	fed	adult	EDTA/DTT		29.33	0.336	
4	sgc	pooled	fed	adult	EDTA/DTT(b)		0.00	0.196	N/D
4	sgc	pooled	fed	adult	EDTA/DTT(b)		2.83	0.221	
4	sgc	pooled	fed	adult	EDTA/DTT(b)		16.92	0.313	
4	sgc	pooled	fed	adult	EDTA/DTT(b)		29.33	0.343	
4	gut	pooled	fed	adult	EDTA/DTT(b)		0.00	0.197	N/D
4	gut	pooled	fed	adult	EDTA/DTT(b)		2.83	0.229	
4	gut	pooled	fed	adult	EDTA/DTT(b)		16.92	0.301	
4	gut	pooled	fed	adult	EDTA/DTT(b)		29.33	0.327	
4	tryp	n/a	fed	n/a			0.00	0.212	0.010
4	tryp	n/a	fed	n/a			2.83	0.937	
4	tryp	n/a	fed	n/a			16.92	1.946	
4	tryp	n/a	fed	n/a			29.33	2.066	
5	blank	n/a	fed	n/a			0.00	0.208	0.000
5	blank	n/a	fed	n/a			1.42	0.224	
5	blank	n/a	fed	n/a			16.75	0.291	
5	blank	n/a	fed	n/a			22.50	0.306	
5	sgc	pooled	fed	adult			0.00	0.224	N/D
5	sgc	pooled	fed	adult			1.42	0.233	
5	sgc	pooled	fed	adult			16.75	0.404	
5	sgc	pooled	fed	adult			22.50	0.484	
5	gut	pooled	fed	adult			0.00	0.221	N/D
5	gut	pooled	fed	adult			1.42	0.239	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
5	gut	pooled	fed	adult			16.75	0.339	
5	gut	pooled	fed	adult			22.50	0.412	
5	sgc	pooled	fed	adult	EDTA/DTT		0.00	0.209	N/D
5	sgc	pooled	fed	adult	EDTA/DTT		1.42	0.217	
5	sgc	pooled	fed	adult	EDTA/DTT		16.75	0.355	
5	sgc	pooled	fed	adult	EDTA/DTT		22.50	0.411	
5	gut	pooled	fed	adult	EDTA/DTT		0.00	0.216	N/D
5	gut	pooled	fed	adult	EDTA/DTT		1.42	0.224	
5	gut	pooled	fed	adult	EDTA/DTT		16.75	0.305	
5	gut	pooled	fed	adult	EDTA/DTT		22.50	0.347	
5	sgc	pooled	fed	adult	EDTA/DTT(b)		0.00	0.202	N/D
5	sgc	pooled	fed	adult	EDTA/DTT(b)		1.42	0.208	
5	sgc	pooled	fed	adult	EDTA/DTT(b)		16.75	0.334	
5	sgc	pooled	fed	adult	EDTA/DTT(b)		22.50	0.384	
5	gut	pooled	fed	adult	EDTA/DTT(b)		0.00	0.208	N/D
5	gut	pooled	fed	adult	EDTA/DTT(b)		1.42	0.217	
5	gut	pooled	fed	adult	EDTA/DTT(b)		16.75	0.293	
5	gut	pooled	fed	adult	EDTA/DTT(b)		22.50	0.321	
5	sgc	female	fed	adult			0.00	0.193	0.854
5	sgc	female	fed	adult			1.42	0.224	
5	sgc	female	fed	adult			16.75	0.456	
5	sgc	female	fed	adult			22.50	0.542	
5	gut	female	fed	adult			0.00	0.203	2.213
5	gut	female	fed	adult			1.42	0.224	
5	gut	female	fed	adult			16.75	0.372	
5	gut	female	fed	adult			22.50	0.453	
5	tryp	n/a	fed	n/a			0.00	0.208	0.010
5	tryp	n/a	fed	n/a			1.42	0.703	
5	tryp	n/a	fed	n/a			16.75	1.995	
5	tryp	n/a	fed	n/a			22.50	2.189	
6	blank	n/a	fed	n/a			0.00	0.197	0.000
6	blank	n/a	fed	n/a			1.08	0.200	
6	blank	n/a	fed	n/a			5.13	0.218	
6	blank	n/a	fed	n/a			19.83	0.272	
6	sgc	female	fed	adult			0.00	0.196	0.657
6	sgc	female	fed	adult			1.08	0.204	
6	sgc	female	fed	adult			5.13	0.263	
6	sgc	female	fed	adult			19.83	0.455	
6	gut	female	fed	adult			0.00	0.202	2.377
6	gut	female	fed	adult			1.08	0.215	
6	gut	female	fed	adult			5.13	0.239	
6	gut	female	fed	adult			19.83	0.332	
6	tryp	n/a	fed	n/a			0.00	0.203	0.010
6	tryp	n/a	fed	n/a			1.08	0.439	
6	tryp	n/a	fed	n/a			5.13	0.770	
6	tryp	n/a	fed	n/a			19.83	1.519	
7	blank	n/a	fed	n/a			0.00	0.251	0.000
7	blank	n/a	fed	n/a			1.50	0.248	
7	blank	n/a	fed	n/a			2.92	0.234	
7	blank	n/a	fed	n/a			17.75	0.266	
7	sgc	female	fed	adult			0.00	0.258	0.788
7	sgc	female	fed	adult			1.50	0.267	
7	sgc	female	fed	adult			2.92	0.299	
7	sgc	female	fed	adult			17.75	0.622	
7	gut	female	fed	adult			0.00	0.252	1.150
7	gut	female	fed	adult			1.50	0.240	
7	gut	female	fed	adult			2.92	0.242	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
7	gut	female	fed	adult			17.75	0.281	
7	sgc	female	fed	adult		AEBSF	0.00	0.252	0.788
7	sgc	female	fed	adult		AEBSF	1.50	0.242	
7	sgc	female	fed	adult		AEBSF	2.92	0.266	
7	sgc	female	fed	adult		AEBSF	17.75	0.409	
7	gut	female	fed	adult		AEBSF	0.00	0.254	1.150
7	gut	female	fed	adult		AEBSF	1.50	0.244	
7	gut	female	fed	adult		AEBSF	2.92	0.232	
7	gut	female	fed	adult		AEBSF	17.75	0.272	
7	tryp	n/a	fed	n/a			0.00	0.261	0.010
7	tryp	n/a	fed	n/a			1.50	0.693	
7	tryp	n/a	fed	n/a			2.92	0.815	
7	tryp	n/a	fed	n/a			17.75	1.577	
8	blank	n/a	fed	n/a			0.00	0.278	0.000
8	blank	n/a	fed	n/a			17.50	0.284	
8	blank	n/a	fed	n/a			19.00	0.280	
8	blank	n/a	fed	n/a			22.00	0.313	
8	sgc	male	fed	adult			0.00	0.280	0.807
8	sgc	male	fed	adult			17.50	0.515	
8	sgc	male	fed	adult			19.00	0.528	
8	sgc	male	fed	adult			22.00	0.679	
8	gut	male	fed	adult			0.00	0.300	1.710
8	gut	male	fed	adult			17.50	0.351	
8	gut	male	fed	adult			19.00	0.346	
8	gut	male	fed	adult			22.00	0.402	
8	sgc	male	fed	adult		AEBSF	0.00	0.286	0.807
8	sgc	male	fed	adult		AEBSF	17.50	0.389	
8	sgc	male	fed	adult		AEBSF	19.00	0.398	
8	sgc	male	fed	adult		AEBSF	22.00	0.477	
8	gut	male	fed	adult		AEBSF	0.00	0.280	1.710
8	gut	male	fed	adult		AEBSF	17.50	0.336	
8	gut	male	fed	adult		AEBSF	19.00	0.336	
8	gut	male	fed	adult		AEBSF	22.00	0.388	
8	tryp	n/a	fed	n/a			0.00	0.282	1.010
8	tryp	n/a	fed	n/a			17.50	0.765	
8	tryp	n/a	fed	n/a			19.00	0.775	
8	tryp	n/a	fed	n/a			22.00	0.944	
8	tryp	n/a	fed	n/a		AEBSF	0.00	0.275	0.010
8	tryp	n/a	fed	n/a		AEBSF	17.50	0.330	
8	tryp	n/a	fed	n/a		AEBSF	19.00	0.328	
8	tryp	n/a	fed	n/a		AEBSF	22.00	0.403	
9	sgc	male	fed	adult			0.00	0.278	0.825
9	sgc	male	fed	adult			17.50	0.530	
9	sgc	male	fed	adult			19.00	0.556	
9	sgc	male	fed	adult			22.00	0.667	
9	gut	male	fed	adult			0.00	0.284	1.670
9	gut	male	fed	adult			17.50	0.366	
9	gut	male	fed	adult			19.00	0.373	
9	gut	male	fed	adult			22.00	0.410	
9	sgc	male	fed	adult		AEBSF	0.00	0.279	0.825
9	sgc	male	fed	adult		AEBSF	17.50	0.394	
9	sgc	male	fed	adult		AEBSF	19.00	0.411	
9	sgc	male	fed	adult		AEBSF	22.00	0.480	
9	gut	male	fed	adult		AEBSF	0.00	0.276	1.670
9	gut	male	fed	adult		AEBSF	17.50	0.331	
9	gut	male	fed	adult		AEBSF	19.00	0.362	
9	gut	male	fed	adult		AEBSF	22.00	0.382	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
10	blank	n/a	fed	n/a			0.00	0.265	0.000
10	blank	n/a	fed	n/a			1.50	0.282	
10	blank	n/a	fed	n/a			18.50	0.391	
10	blank	n/a	fed	n/a			21.08	0.413	
10	sgc	male	fed	adult			0.00	0.274	0.294
10	sgc	male	fed	adult			1.50	0.298	
10	sgc	male	fed	adult			18.50	0.413	
10	sgc	male	fed	adult			21.08	0.436	
10	gut	male	fed	adult			0.00	0.278	1.250
10	gut	male	fed	adult			1.50	0.289	
10	gut	male	fed	adult			18.50	0.414	
10	gut	male	fed	adult			21.08	0.421	
10	tryp	n/a	fed	n/a			0.00	0.279	0.010
10	tryp	n/a	fed	n/a			1.50	0.765	
10	tryp	n/a	fed	n/a			18.50	2.012	
10	tryp	n/a	fed	n/a			21.08	2.190	
11	blank	n/a	fed	n/a			0.00	0.216	0.000
11	blank	n/a	fed	n/a			4.25	0.224	
11	blank	n/a	fed	n/a			16.50	0.269	
11	blank	n/a	fed	n/a			22.75	0.285	
11	sgc	male	fed	adult			0.00	0.228	0.882
11	sgc	male	fed	adult			4.25	0.327	
11	sgc	male	fed	adult			16.50	0.647	
11	sgc	male	fed	adult			22.75	0.810	
11	gut	male	fed	adult			0.00	0.229	1.785
11	gut	male	fed	adult			4.25	0.225	
11	gut	male	fed	adult			16.50	0.279	
11	gut	male	fed	adult			22.75	0.354	
11	sgc	male	fed	adult		antipain	0.00	0.234	0.882
11	sgc	male	fed	adult		antipain	4.25	0.298	
11	sgc	male	fed	adult		antipain	16.50	0.516	
11	sgc	male	fed	adult		antipain	22.75	0.674	
11	gut	male	fed	adult		antipain	0.00	0.236	1.785
11	gut	male	fed	adult		antipain	4.25	0.222	
11	gut	male	fed	adult		antipain	16.50	0.277	
11	gut	male	fed	adult		antipain	22.75	0.348	
11	sgc	female	fed	adult			0.00	0.230	1.421
11	sgc	female	fed	adult			4.25	0.324	
11	sgc	female	fed	adult			16.50	0.705	
11	sgc	female	fed	adult			22.75	0.938	
11	gut	female	fed	adult			0.00	0.227	2.487
11	gut	female	fed	adult			4.25	0.210	
11	gut	female	fed	adult			16.50	0.278	
11	gut	female	fed	adult			22.75	0.351	
11	sgc	female	fed	adult		antipain	0.00	0.236	1.421
11	sgc	female	fed	adult		antipain	4.25	0.267	
11	sgc	female	fed	adult		antipain	16.50	0.518	
11	sgc	female	fed	adult		antipain	22.75	0.668	
11	gut	female	fed	adult		antipain	0.00	0.234	2.487
11	gut	female	fed	adult		antipain	4.25	0.208	
11	gut	female	fed	adult		antipain	16.50	0.280	
11	gut	female	fed	adult		antipain	22.75	0.341	
12	blank	n/a	fed	n/a			0.00	0.253	0.000
12	blank	n/a	fed	n/a			2.67	0.243	
12	blank	n/a	fed	n/a			4.50	0.250	
12	blank	n/a	fed	n/a			20.67	0.316	
12	sgc	male	fed	adult			0.00	0.268	0.377

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
12	sgc	male	fed	adult			2.67	0.308	
12	sgc	male	fed	adult			4.50	0.370	
12	sgc	male	fed	adult			20.67	0.688	
12	gut	male	fed	adult			0.00	0.274	1.085
12	gut	male	fed	adult			2.67	0.261	
12	gut	male	fed	adult			4.50	0.300	
12	gut	male	fed	adult			20.67	0.467	
12	sgc	male	fed	adult		antipain	0.00	0.278	0.377
12	sgc	male	fed	adult		antipain	2.67	0.304	
12	sgc	male	fed	adult		antipain	4.50	0.346	
12	sgc	male	fed	adult		antipain	20.67	0.627	
12	gut	male	fed	adult		antipain	0.00	0.267	1.085
12	gut	male	fed	adult		antipain	2.67	0.262	
12	gut	male	fed	adult		antipain	4.50	0.280	
12	gut	male	fed	adult		antipain	20.67	0.398	
12	sgc	female	fed	adult			0.00	0.263	0.611
12	sgc	female	fed	adult			2.67	0.278	
12	sgc	female	fed	adult			4.50	0.326	
12	sgc	female	fed	adult			20.67	0.699	
12	gut	female	fed	adult			0.00	0.264	1.581
12	gut	female	fed	adult			2.67	0.248	
12	gut	female	fed	adult			4.50	0.282	
12	gut	female	fed	adult			20.67	0.336	
12	sgc	female	fed	adult		antipain	0.00	0.270	0.611
12	sgc	female	fed	adult		antipain	2.67	0.257	
12	sgc	female	fed	adult		antipain	4.50	0.281	
12	sgc	female	fed	adult		antipain	20.67	0.467	
12	gut	female	fed	adult		antipain	0.00	0.264	1.581
12	gut	female	fed	adult		antipain	2.67	0.245	
12	gut	female	fed	adult		antipain	4.50	0.266	
12	gut	female	fed	adult		antipain	20.67	0.309	
12	tryp	n/a	fed	n/a			0.00	0.270	0.010
12	tryp	n/a	fed	n/a			2.67	0.325	
12	tryp	n/a	fed	n/a			4.50	0.380	
12	tryp	n/a	fed	n/a			20.67	0.854	
12	tryp	n/a	fed	n/a		antipain	0.00	0.280	0.010
12	tryp	n/a	fed	n/a		antipain	2.67	0.251	
12	tryp	n/a	fed	n/a		antipain	4.50	0.277	
12	tryp	n/a	fed	n/a		antipain	20.67	0.445	
13	blank	n/a	fed	n/a			0.00	0.301	0.000
13	blank	n/a	fed	n/a			1.18	0.268	
13	blank	n/a	fed	n/a			15.58	0.322	
13	blank	n/a	fed	n/a			20.67	0.350	
13	sgc	male	fed	adult			0.00	0.314	0.574
13	sgc	male	fed	adult			1.18	0.318	
13	sgc	male	fed	adult			15.58	0.628	
13	sgc	male	fed	adult			20.67	0.757	
13	gut	male	fed	adult			0.00	0.307	1.351
13	gut	male	fed	adult			1.18	0.296	
13	gut	male	fed	adult			15.58	0.364	
13	gut	male	fed	adult			20.67	0.506	
13	sgc	male	fed	adult		antipain(b)	0.00	0.314	0.574
13	sgc	male	fed	adult		antipain(b)	1.18	0.308	
13	sgc	male	fed	adult		antipain(b)	15.58	0.478	
13	sgc	male	fed	adult		antipain(b)	20.67	0.555	
13	gut	male	fed	adult		antipain(b)	0.00	0.312	1.351
13	gut	male	fed	adult		antipain(b)	1.18	0.296	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
13	gut	male	fed	adult		antipain(b)	15.58	0.352	
13	gut	male	fed	adult		antipain(b)	20.67	0.518	
13	sgc	female	fed	adult			0.00	0.319	0.845
13	sgc	female	fed	adult			1.18	0.316	
13	sgc	female	fed	adult			15.58	0.618	
13	sgc	female	fed	adult			20.67	0.803	
13	gut	female	fed	adult			0.00	0.316	1.673
13	gut	female	fed	adult			1.18	0.273	
13	gut	female	fed	adult			15.58	0.337	
13	gut	female	fed	adult			20.67	0.392	
13	sgc	female	fed	adult		antipain(b)	0.00	0.318	0.845
13	sgc	female	fed	adult		antipain(b)	1.18	0.295	
13	sgc	female	fed	adult		antipain(b)	15.58	0.484	
13	sgc	female	fed	adult		antipain(b)	20.67	0.554	
13	gut	female	fed	adult		antipain(b)	0.00	0.319	1.673
13	gut	female	fed	adult		antipain(b)	1.18	0.289	
13	gut	female	fed	adult		antipain(b)	15.58	0.338	
13	gut	female	fed	adult		antipain(b)	20.67	0.387	
13	tryp	n/a	fed	n/a			0.00	0.341	0.010
13	tryp	n/a	fed	n/a			1.18	0.413	
13	tryp	n/a	fed	n/a			15.58	0.894	
13	tryp	n/a	fed	n/a		antipain(b)	0.00	0.337	0.010
13	tryp	n/a	fed	n/a		antipain(b)	1.18	0.312	
13	tryp	n/a	fed	n/a		antipain(b)	15.58	0.437	
14	blank	n/a	fed	n/a			0.00	0.268	0.000
14	blank	n/a	fed	n/a			3.92	0.304	
14	blank	n/a	fed	n/a			18.33	0.374	
14	blank	n/a	fed	n/a			23.00	0.382	
14	sgc	female	fed	adult			0.00	0.285	0.841
14	sgc	female	fed	adult			3.92	0.380	
14	sgc	female	fed	adult			18.33	0.864	
14	sgc	female	fed	adult			23.00	1.051	
14	gut	female	fed	adult			0.00	0.275	2.392
14	gut	female	fed	adult			3.92	0.325	
14	gut	female	fed	adult			18.33	0.440	
14	gut	female	fed	adult			23.00	0.484	
14	sgc	female	fed	adult		E-64	0.00	0.289	0.841
14	sgc	female	fed	adult		E-64	3.92	0.402	
14	sgc	female	fed	adult		E-64	18.33	0.869	
14	sgc	female	fed	adult		E-64	23.00	1.061	
14	gut	female	fed	adult		E-64	0.00	0.282	2.392
14	gut	female	fed	adult		E-64	3.92	0.322	
14	gut	female	fed	adult		E-64	18.33	0.433	
14	gut	female	fed	adult		E-64	23.00	0.472	
14	saliva	pooled	fed	adult			0.00	0.271	N/D
14	saliva	pooled	fed	adult			3.92	0.310	
14	saliva	pooled	fed	adult			18.33	0.396	
14	saliva	pooled	fed	adult			23.00	0.485	
14	saliva	pooled	fed	adult		E-64	0.00	0.279	N/D
14	saliva	pooled	fed	adult		E-64	3.92	0.311	
14	saliva	pooled	fed	adult		E-64	18.33	0.389	
14	saliva	pooled	fed	adult		E-64	23.00	0.483	
14	tryp	n/a	fed	n/a			0.00	0.280	0.010
14	tryp	n/a	fed	n/a			3.92	0.493	
14	tryp	n/a	fed	n/a			18.33	0.931	
14	tryp	n/a	fed	n/a			23.00	1.110	
15	blank	n/a	fed	n/a			0.00	0.210	0.000

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
15	blank	n/a	fed	n/a			16.83	0.265	
15	blank	n/a	fed	n/a			19.08	0.284	
15	blank	n/a	fed	n/a			22.08	0.360	
15	sgc	female	fed	adult			0.00	0.214	0.394
15	sgc	female	fed	adult			16.83	0.442	
15	sgc	female	fed	adult			19.08	0.635	
15	sgc	female	fed	adult			22.08	1.035	
15	gut	female	fed	adult			0.00	0.223	2.044
15	gut	female	fed	adult			16.83	0.414	
15	gut	female	fed	adult			19.08	0.616	
15	gut	female	fed	adult			22.08	0.953	
15	sgc	female	fed	adult		E-64(b)	0.00	0.211	0.394
15	sgc	female	fed	adult		E-64(b)	16.83	0.501	
15	sgc	female	fed	adult		E-64(b)	19.08	0.520	
15	sgc	female	fed	adult		E-64(b)	22.08	0.797	
15	gut	female	fed	adult		E-64(b)	0.00	0.230	2.044
15	gut	female	fed	adult		E-64(b)	16.83	0.378	
15	gut	female	fed	adult		E-64(b)	19.08	0.515	
15	gut	female	fed	adult		E-64(b)	22.08	0.794	
15	saliva	pooled	fed	adult			0.00	0.224	N/D
15	saliva	pooled	fed	adult			16.83	0.381	
15	saliva	pooled	fed	adult			19.08	0.473	
15	saliva	pooled	fed	adult			22.08	0.738	
15	saliva	pooled	fed	adult		E-64(b)	0.00	0.224	N/D
15	saliva	pooled	fed	adult		E-64(b)	16.83	0.325	
15	saliva	pooled	fed	adult		E-64(b)	19.08	0.358	
15	saliva	pooled	fed	adult		E-64(b)	22.08	0.457	
16	blank	n/a	fed	n/a			0.00	0.288	0.000
16	blank	n/a	fed	n/a			2.25	0.264	
16	blank	n/a	fed	n/a			19.67	0.361	
16	blank	n/a	fed	n/a			22.50	0.515	
16	sgc	male	fed	adult			0.00	0.302	0.177
16	sgc	male	fed	adult			2.25	0.284	
16	sgc	male	fed	adult			19.67	0.623	
16	sgc	male	fed	adult			22.50	0.822	
16	gut	male	fed	adult			0.00	0.313	1.079
16	gut	male	fed	adult			2.25	0.285	
16	gut	male	fed	adult			19.67	0.642	
16	gut	male	fed	adult			22.50	0.822	
16	sgc	male	fed	adult		E-64(c)	0.00	0.313	0.177
16	sgc	male	fed	adult		E-64(c)	2.25	0.279	
16	sgc	male	fed	adult		E-64(c)	19.67	0.482	
16	sgc	male	fed	adult		E-64(c)	22.50	0.633	
16	gut	male	fed	adult		E-64(c)	0.00	0.311	1.079
16	gut	male	fed	adult		E-64(c)	2.25	0.284	
16	gut	male	fed	adult		E-64(c)	19.67	0.582	
16	gut	male	fed	adult		E-64(c)	22.50	0.755	
16	sgc	female	fed	adult			0.00	0.312	0.512
16	sgc	female	fed	adult			2.25	0.357	
16	sgc	female	fed	adult			19.67	1.192	
16	sgc	female	fed	adult			22.50	1.459	
16	gut	female	fed	adult			0.00	0.331	2.048
16	gut	female	fed	adult			2.25	0.318	
16	gut	female	fed	adult			19.67	0.734	
16	gut	female	fed	adult			22.50	0.984	
16	sgc	female	fed	adult		E-64(c)	0.00	0.317	0.512
16	sgc	female	fed	adult		E-64(c)	2.25	0.342	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
16	sgc	female	fed	adult		E-64(c)	19.67	0.981	
16	sgc	female	fed	adult		E-64(c)	22.50	1.209	
16	gut	female	fed	adult		E-64(c)	0.00	0.331	2.048
16	gut	female	fed	adult		E-64(c)	2.25	0.302	
16	gut	female	fed	adult		E-64(c)	19.67	0.630	
16	gut	female	fed	adult		E-64(c)	22.50	0.892	
17	blank	n/a	fed	n/a			0.00	0.193	0.000
17	blank	n/a	fed	n/a			17.58	0.284	
17	blank	n/a	fed	n/a			22.00	0.339	
17	blank	n/a	fed	n/a			23.50	0.335	
17	sgc	male	fed	adult			0.00	0.207	0.637
17	sgc	male	fed	adult			17.58	0.501	
17	sgc	male	fed	adult			22.00	0.598	
17	sgc	male	fed	adult			23.50	0.707	
17	sgc	pooled	fed	nymph			0.00	0.215	1.368
17	sgc	pooled	fed	nymph			17.58	0.809	
17	sgc	pooled	fed	nymph			22.00	1.136	
17	sgc	pooled	fed	nymph			23.50	1.332	
17	gut	pooled	fed	nymph			0.00	0.238	4.590
17	gut	pooled	fed	nymph			17.58	0.325	
17	gut	pooled	fed	nymph			22.00	0.485	
17	gut	pooled	fed	nymph			23.50	0.628	
18	sgc	pooled	fed	nymph			0.00	0.229	0.548
18	sgc	pooled	fed	nymph			17.58	0.597	
18	sgc	pooled	fed	nymph			22.00	0.703	
18	sgc	pooled	fed	nymph			23.50	0.792	
18	gut	pooled	fed	nymph			0.00	0.238	2.110
18	gut	pooled	fed	nymph			17.58	0.333	
18	gut	pooled	fed	nymph			22.00	0.416	
18	gut	pooled	fed	nymph			23.50	0.562	
19	blank	n/a	fed	n/a			0.00	0.222	0.000
19	blank	n/a	fed	n/a			16.75	0.236	
19	blank	n/a	fed	n/a			20.75	0.270	
19	sgc	male	fed	adult			0.00	0.251	0.329
19	sgc	male	fed	adult			16.75	0.557	
19	sgc	male	fed	adult			20.75	0.703	
19	sgc	male	fed	adult			22.42	0.728	
19	gut	male	fed	adult			0.00	0.251	1.385
19	gut	male	fed	adult			16.75	0.307	
19	gut	male	fed	adult			20.75	0.329	
19	gut	male	fed	adult			22.42	0.368	
19	sgc	pooled	fed	nymph			0.00	0.238	0.336
19	sgc	pooled	fed	nymph			16.75	0.951	
19	sgc	pooled	fed	nymph			20.75	1.111	
19	sgc	pooled	fed	nymph			22.42	1.112	
19	gut	pooled	fed	nymph			0.00	0.254	0.845
19	gut	pooled	fed	nymph			16.75	0.274	
19	gut	pooled	fed	nymph			20.75	0.289	
19	gut	pooled	fed	nymph			22.42	0.300	
19	saliva	pooled	fed	adult			0.00	0.246	N/D
19	saliva	pooled	fed	adult			16.75	0.306	
19	saliva	pooled	fed	adult			20.75	0.501	
19	saliva	pooled	fed	adult			22.42	0.588	
19	saliva	pooled	fed	adult		AEBSF	0.00	0.227	N/D
19	saliva	pooled	fed	adult		AEBSF	16.75	0.244	
19	saliva	pooled	fed	adult		AEBSF	20.75	0.247	
19	saliva	pooled	fed	adult		AEBSF	22.42	0.292	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
19	tryp	n/a	fed	n/a			0.00	0.254	0.010
19	tryp	n/a	fed	n/a			16.75	1.519	
19	tryp	n/a	fed	n/a			20.75	1.646	
19	tryp	n/a	fed	n/a			22.42	1.654	
19	tryp	n/a	fed	n/a		AEBSF	0.00	0.266	0.010
19	tryp	n/a	fed	n/a		AEBSF	16.75	0.246	
19	tryp	n/a	fed	n/a		AEBSF	20.75	0.268	
19	tryp	n/a	fed	n/a		AEBSF	22.42	0.296	
20	sgc	pooled	fed	nymph			0.00	0.242	0.451
20	sgc	pooled	fed	nymph			16.75	0.529	
20	sgc	pooled	fed	nymph			20.75	0.648	
20	sgc	pooled	fed	nymph			22.42	0.685	
20	gut	pooled	fed	nymph			0.00	0.256	2.828
20	gut	pooled	fed	nymph			16.75	0.299	
20	gut	pooled	fed	nymph			20.75	0.319	
20	gut	pooled	fed	nymph			22.42	0.332	
21	blank	n/a	fed	n/a			0.00	0.212	0.000
21	blank	n/a	fed	n/a			2.83	0.223	
21	blank	n/a	fed	n/a			5.42	0.238	
21	blank	n/a	fed	n/a			20.50	0.285	
21	sgc	female	fed	adult			0.00	0.223	0.805
21	sgc	female	fed	adult			2.83	0.262	
21	sgc	female	fed	adult			5.42	0.309	
21	sgc	female	fed	adult			20.50	0.534	
21	gut	female	fed	adult			0.00	0.222	1.676
21	gut	female	fed	adult			2.83	0.244	
21	gut	female	fed	adult			5.42	0.272	
21	gut	female	fed	adult			20.50	0.390	
21	sgc	female	fed	adult		AEBSF/E-64	0.00	0.225	0.805
21	sgc	female	fed	adult		AEBSF/E-64	2.83	0.233	
21	sgc	female	fed	adult		AEBSF/E-64	5.42	0.243	
21	sgc	female	fed	adult		AEBSF/E-64	20.50	0.300	
21	gut	female	fed	adult		AEBSF/E-64	0.00	0.226	1.676
21	gut	female	fed	adult		AEBSF/E-64	2.83	0.235	
21	gut	female	fed	adult		AEBSF/E-64	5.42	0.246	
21	gut	female	fed	adult		AEBSF/E-64	20.50	0.283	
21	saliva	pooled	fed	adult			0.00	0.222	0.240
21	saliva	pooled	fed	adult			2.83	0.238	
21	saliva	pooled	fed	adult			5.42	0.267	
21	saliva	pooled	fed	adult			20.50	0.789	
21	saliva	pooled	fed	adult		AEBSF	0.00	0.229	0.240
21	saliva	pooled	fed	adult		AEBSF	2.83	0.231	
21	saliva	pooled	fed	adult		AEBSF	5.42	0.255	
21	saliva	pooled	fed	adult		AEBSF	20.50	0.290	
21	saliva	pooled	fed	adult		AEBSF/E-64	0.00	0.224	0.240
21	saliva	pooled	fed	adult		AEBSF/E-64	2.83	0.234	
21	saliva	pooled	fed	adult		AEBSF/E-64	5.42	0.241	
21	saliva	pooled	fed	adult		AEBSF/E-64	20.50	0.287	
21	tryp	n/a	fed	n/a			0.00	0.223	0.010
21	tryp	n/a	fed	n/a			2.83	0.760	
21	tryp	n/a	fed	n/a			5.42	1.113	
21	tryp	n/a	fed	n/a			20.50	1.828	
21	tryp	n/a	fed	n/a		AEBSF/E-64	0.00	0.227	0.010
21	tryp	n/a	fed	n/a		AEBSF/E-64	2.83	0.240	
21	tryp	n/a	fed	n/a		AEBSF/E-64	5.42	0.255	
21	tryp	n/a	fed	n/a		AEBSF/E-64	20.50	0.379	
22	blank	n/a	fed	n/a			0.00	0.238	0.000

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
22	blank	n/a	fed	n/a			17.17	0.306	
22	blank	n/a	fed	n/a			20.17	0.320	
22	blank	n/a	fed	n/a			22.33	0.342	
22	sgc	male	fed	adult			0.00	0.225	0.311
22	sgc	male	fed	adult			17.17	0.603	
22	sgc	male	fed	adult			20.17	0.676	
22	sgc	male	fed	adult			22.33	0.739	
22	gut	male	fed	adult			0.00	0.244	0.944
22	gut	male	fed	adult			17.17	0.330	
22	gut	male	fed	adult			20.17	0.374	
22	gut	male	fed	adult			22.33	0.505	
22	sgc	male	fed	adult		E-64(d)	0.00	0.231	0.311
22	sgc	male	fed	adult		E-64(d)	17.17	0.570	
22	sgc	male	fed	adult		E-64(d)	20.17	0.635	
22	sgc	male	fed	adult		E-64(d)	22.33	0.722	
22	gut	male	fed	adult		E-64(d)	0.00	0.235	0.944
22	gut	male	fed	adult		E-64(d)	17.17	0.335	
22	gut	male	fed	adult		E-64(d)	20.17	0.370	
22	gut	male	fed	adult		E-64(d)	22.33	0.501	
22	saliva	pooled	fed	adult			0.00	0.245	0.081
22	saliva	pooled	fed	adult			17.17	0.484	
22	saliva	pooled	fed	adult			20.17	0.729	
22	saliva	pooled	fed	adult			22.33	1.043	
22	saliva	pooled	fed	adult		E-64(d)	0.00	0.249	0.081
22	saliva	pooled	fed	adult		E-64(d)	17.17	0.379	
22	saliva	pooled	fed	adult		E-64(d)	20.17	0.535	
22	saliva	pooled	fed	adult		E-64(d)	22.33	0.739	
22	saliva	pooled	fed	adult		AEBSF/E-64(b)	0.00	0.242	0.081
22	saliva	pooled	fed	adult		AEBSF/E-64(b)	17.17	0.292	
22	saliva	pooled	fed	adult		AEBSF/E-64(b)	20.17	0.322	
22	saliva	pooled	fed	adult		AEBSF/E-64(b)	22.33	0.353	
23	blank	pooled	n/a	n/a			0.00	0.248	0.000
23	blank	pooled	n/a	n/a			3.67	0.259	
23	blank	pooled	n/a	n/a			21.50	0.338	
23	blank	pooled	n/a	n/a			23.50	0.357	
23	sgc	pooled	fed	adult			0.00	0.261	N/D
23	sgc	pooled	fed	adult			3.67	0.290	
23	sgc	pooled	fed	adult			21.50	0.575	
23	sgc	pooled	fed	adult			23.50	0.613	
23	gut	pooled	fed	adult			0.00	0.268	N/D
23	gut	pooled	fed	adult			3.67	0.294	
23	gut	pooled	fed	adult			21.50	0.395	
23	gut	pooled	fed	adult			23.50	0.415	
23	sgc	pooled	starved	adult			0.00	0.263	N/D
23	sgc	pooled	starved	adult			3.67	0.308	
23	sgc	pooled	starved	adult			21.50	0.720	
23	sgc	pooled	starved	adult			23.50	0.728	
23	gut	pooled	starved	adult			0.00	0.267	N/D
23	gut	pooled	starved	adult			3.67	0.278	
23	gut	pooled	starved	adult			21.50	0.341	
23	gut	pooled	starved	adult			23.50	0.371	
24	sgc	pooled	fed	adult			0.00	0.259	N/D
24	sgc	pooled	fed	adult			3.67	0.278	
24	sgc	pooled	fed	adult			21.50	0.381	
24	sgc	pooled	fed	adult			23.50	0.388	
24	gut	pooled	fed	adult			0.00	0.260	N/D
24	gut	pooled	fed	adult			3.67	0.301	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
24	gut	pooled	fed	adult			21.50	0.443	
24	gut	pooled	fed	adult			23.50	0.454	
24	sgc	pooled	starved	adult			0.00	0.268	N/D
24	sgc	pooled	starved	adult			3.67	0.286	
24	sgc	pooled	starved	adult			21.50	0.521	
24	sgc	pooled	starved	adult			23.50	0.560	
24	gut	pooled	starved	adult			0.00	0.268	N/D
24	gut	pooled	starved	adult			3.67	0.279	
24	gut	pooled	starved	adult			21.50	0.345	
24	gut	pooled	starved	adult			23.50	0.340	
25	blank	n/a	n/a	n/a			0.00	0.198	0.000
25	blank	n/a	n/a	n/a			3.00	0.221	
25	blank	n/a	n/a	n/a			24.00	0.360	
25	sgc	pooled	fed	adult			0.00	0.208	0.771
25	sgc	pooled	fed	adult			3.00	0.308	
25	sgc	pooled	fed	adult			24.00	0.798	
25	gut	pooled	fed	adult			0.00	0.220	1.041
25	gut	pooled	fed	adult			3.00	0.240	
25	gut	pooled	fed	adult			24.00	0.392	
25	sgc	pooled	starved	adult			0.00	0.212	0.570
25	sgc	pooled	starved	adult			3.00	0.275	
25	sgc	pooled	starved	adult			24.00	0.672	
25	gut	pooled	starved	adult			0.00	0.225	0.674
25	gut	pooled	starved	adult			3.00	0.240	
25	gut	pooled	starved	adult			24.00	0.377	
26	sgc	pooled	fed	adult			0.00	0.220	0.599
26	sgc	pooled	fed	adult			3.00	0.393	
26	sgc	pooled	fed	adult			24.00	0.966	
26	gut	pooled	fed	adult			0.00	0.229	0.899
26	gut	pooled	fed	adult			3.00	0.250	
26	gut	pooled	fed	adult			24.00	0.400	
26	sgc	pooled	starved	adult			0.00	0.221	0.522
26	sgc	pooled	starved	adult			3.00	0.266	
26	sgc	pooled	starved	adult			24.00	0.674	
26	gut	pooled	starved	adult			0.00	0.226	0.727
26	gut	pooled	starved	adult			3.00	0.249	
26	gut	pooled	starved	adult			24.00	0.390	
27	blank	n/a	n/a	n/a			0.00	0.226	0.000
27	blank	n/a	n/a	n/a			3.00	0.242	
27	blank	n/a	n/a	n/a			20.50	0.315	
27	blank	n/a	n/a	n/a			21.00	0.335	
27	sgc	pooled	fed	adult			0.00	0.239	0.420
27	sgc	pooled	fed	adult			3.00	0.268	
27	sgc	pooled	fed	adult			20.50	0.446	
27	sgc	pooled	fed	adult			21.00	0.512	
27	gut	pooled	fed	adult			0.00	0.247	2.015
27	gut	pooled	fed	adult			3.00	0.290	
27	gut	pooled	fed	adult			20.50	0.382	
27	gut	pooled	fed	adult			21.00	0.425	
27	sgc	pooled	starved	adult			0.00	0.231	0.381
27	sgc	pooled	starved	adult			3.00	0.275	
27	sgc	pooled	starved	adult			20.50	0.527	
27	sgc	pooled	starved	adult			21.00	0.603	
27	gut	pooled	starved	adult			0.00	0.245	1.175
27	gut	pooled	starved	adult			3.00	0.274	
27	gut	pooled	starved	adult			20.50	0.740	
27	gut	pooled	starved	adult			21.00	1.027	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
28	blank	n/a	n/a	n/a			0.00	0.236	0.000
28	blank	n/a	n/a	n/a			3.00	0.271	
28	blank	n/a	n/a	n/a			18.50	0.344	
28	blank	n/a	n/a	n/a			24.00	0.340	
28	sgc	pooled	fed	adult			0.00	0.255	0.340
28	sgc	pooled	fed	adult			3.00	0.387	
28	sgc	pooled	fed	adult			18.50	0.715	
28	sgc	pooled	fed	adult			24.00	0.834	
28	gut	pooled	fed	adult			0.00	0.253	0.834
28	gut	pooled	fed	adult			3.00	0.277	
28	gut	pooled	fed	adult			18.50	0.362	
28	gut	pooled	fed	adult			24.00	0.391	
28	sgc	pooled	starved	adult			0.00	0.243	0.372
28	sgc	pooled	starved	adult			3.00	0.325	
28	sgc	pooled	starved	adult			18.50	0.540	
28	sgc	pooled	starved	adult			24.00	0.610	
28	gut	pooled	starved	adult			0.00	0.249	0.872
28	gut	pooled	starved	adult			3.00	0.318	
28	gut	pooled	starved	adult			18.50	0.468	
28	gut	pooled	starved	adult			24.00	0.526	
29	blank	n/a	n/a	n/a			0.00	0.230	0.000
29	blank	n/a	n/a	n/a			3.00	0.340	
29	blank	n/a	n/a	n/a			21.00	0.318	
29	blank	n/a	n/a	n/a			24.00	0.374	
29	sgc	pooled	fed	adult			0.00	0.236	0.366
29	sgc	pooled	fed	adult			3.00	0.283	
29	sgc	pooled	fed	adult			21.00	0.594	
29	sgc	pooled	fed	adult			24.00	0.672	
29	gut	pooled	fed	adult			0.00	0.233	0.165
29	gut	pooled	fed	adult			3.00	0.247	
29	gut	pooled	fed	adult			21.00	0.334	
29	gut	pooled	fed	adult			24.00	0.368	
29	sgc	pooled	starved	adult			0.00	0.220	0.398
29	sgc	pooled	starved	adult			3.00	0.268	
29	sgc	pooled	starved	adult			21.00	0.549	
29	sgc	pooled	starved	adult			24.00	0.627	
29	gut	pooled	starved	adult			0.00	0.232	0.712
29	gut	pooled	starved	adult			3.00	0.242	
29	gut	pooled	starved	adult			21.00	0.371	
29	gut	pooled	starved	adult			24.00	0.394	
30	sgc	pooled	fed	adult			0.00	0.237	0.758
30	sgc	pooled	fed	adult			3.00	0.342	
30	sgc	pooled	fed	adult			21.00	0.881	
30	sgc	pooled	fed	adult			24.00	0.995	
30	gut	pooled	fed	adult			0.00	0.243	1.451
30	gut	pooled	fed	adult			3.00	0.266	
30	gut	pooled	fed	adult			21.00	0.496	
30	gut	pooled	fed	adult			24.00	0.643	
30	sgc	pooled	starved	adult			0.00	0.226	0.462
30	sgc	pooled	starved	adult			3.00	0.276	
30	sgc	pooled	starved	adult			21.00	0.577	
30	sgc	pooled	starved	adult			24.00	0.648	
30	gut	pooled	starved	adult			0.00	0.244	4.165
30	gut	pooled	starved	adult			3.00	0.295	
30	gut	pooled	starved	adult			21.00	0.847	
30	gut	pooled	starved	adult			24.00	1.005	

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
31	blank	n/a	n/a	n/a			0.00	0.284	0.000
31	blank	n/a	n/a	n/a			3.00	0.319	
31	blank	n/a	n/a	n/a			6.75	0.347	
31	blank	n/a	n/a	n/a			22.25	0.431	
31	conts	pooled	fed	adult			0.00	0.228	N/D
31	conts	pooled	fed	adult			3.00	0.288	
31	conts	pooled	fed	adult			6.75	0.364	
31	conts	pooled	fed	adult			22.25	0.461	
31	tryp	n/a	n/a	n/a			0.00	0.318	0.010
31	tryp	n/a	n/a	n/a			3.00	0.544	
31	tryp	n/a	n/a	n/a			6.75	0.728	
31	tryp	n/a	n/a	n/a			22.25	1.216	
32	conts	pooled	fed	adult			0.00	0.308	N/D
32	conts	pooled	fed	adult			3.00	0.378	
32	conts	pooled	fed	adult			6.75	0.474	
32	conts	pooled	fed	adult			22.25	0.755	
33	conts	pooled	fed	adult			0.00	0.302	N/D
33	conts	pooled	fed	adult			3.00	0.351	
33	conts	pooled	fed	adult			6.75	0.415	
33	conts	pooled	fed	adult			22.25	0.583	
34	blank	n/a	n/a	n/a			0.00	0.238	
34	blank	n/a	n/a	n/a			3.00	0.249	
34	blank	n/a	n/a	n/a			8.33	0.276	
34	blank	n/a	n/a	n/a			23.00	0.372	
34	conts	pooled	fed	adult			0.00	0.260	N/D
34	conts	pooled	fed	adult			3.00	0.263	
34	conts	pooled	fed	adult			8.33	0.285	
34	conts	pooled	fed	adult			23.00	0.392	
34	tryp	n/a	n/a	n/a			0.00	0.263	0.010
34	tryp	n/a	n/a	n/a			3.00	0.832	
34	tryp	n/a	n/a	n/a			8.33	1.301	
34	tryp	n/a	n/a	n/a			23.00	1.958	
35	conts	pooled	fed	adult			0.00	0.254	N/D
35	conts	pooled	fed	adult			3.00	0.297	
35	conts	pooled	fed	adult			8.33	0.359	
35	conts	pooled	fed	adult			23.00	0.510	
36	conts	pooled	fed	adult			0.00	0.264	N/D
36	conts	pooled	fed	adult			3.00	0.292	
36	conts	pooled	fed	adult			8.33	0.340	
36	conts	pooled	fed	adult			23.00	0.463	
37	conts	pooled	fed	adult			0.00	0.261	N/D
37	conts	pooled	fed	adult			3.00	0.279	
37	conts	pooled	fed	adult			8.33	0.312	
37	conts	pooled	fed	adult			23.00	0.443	
38	blank	n/a	n/a	n/a			0.00	0.226	0.000
38	blank	n/a	n/a	n/a			5.00	0.250	
38	blank	n/a	n/a	n/a			11.00	0.286	
38	blank	n/a	n/a	n/a			22.00	0.355	
38	sgc	pooled	fed	adult			0.00	0.239	0.798
38	sgc	pooled	fed	adult			5.00	0.371	
38	sgc	pooled	fed	adult			11.00	0.518	
38	sgc	pooled	fed	adult			22.00	0.860	
38	sgc	pooled	fed	adult		aprotinin	0.00	0.250	0.798
38	sgc	pooled	fed	adult		aprotinin	5.00	0.275	
38	sgc	pooled	fed	adult		aprotinin	11.00	0.308	
38	sgc	pooled	fed	adult		aprotinin	22.00	0.407	
38	gut	pooled	fed	adult			0.00	0.255	1.823

Rep	Sample	Sex	Feed	Age	Activators	Inhibitors	Time	Absorbance	Amt. protein
38	gut	pooled	fed	adult			5.00	0.267	
38	gut	pooled	fed	adult			11.00	0.296	
38	gut	pooled	fed	adult			22.00	0.382	
38	gut	pooled	fed	adult		aprotinin	0.00	0.254	1.823
38	gut	pooled	fed	adult		aprotinin	5.00	0.272	
38	gut	pooled	fed	adult		aprotinin	11.00	0.298	
38	gut	pooled	fed	adult		aprotinin	22.00	0.344	
38	tryp	n/a	n/a	n/a			0.00	0.269	0.010
38	tryp	n/a	n/a	n/a			5.00	0.956	
38	tryp	n/a	n/a	n/a			11.00	1.296	
38	tryp	n/a	n/a	n/a			22.00	1.778	
38	tryp	n/a	n/a	n/a		aprotinin	0.00	0.258	0.010
38	tryp	n/a	n/a	n/a		aprotinin	5.00	0.335	
38	tryp	n/a	n/a	n/a		aprotinin	11.00	0.342	
38	tryp	n/a	n/a	n/a		aprotinin	22.00	0.450	
39	sgc	pooled	fed	adult			0.00	0.249	0.882
39	sgc	pooled	fed	adult			5.00	0.339	
39	sgc	pooled	fed	adult			11.00	0.466	
39	sgc	pooled	fed	adult			22.00	0.739	
39	sgc	pooled	fed	adult		aprotinin	0.00	0.261	0.882
39	sgc	pooled	fed	adult		aprotinin	5.00	0.269	
39	sgc	pooled	fed	adult		aprotinin	11.00	0.299	
39	sgc	pooled	fed	adult		aprotinin	22.00	0.380	
39	gut	pooled	fed	adult			0.00	0.257	1.610
39	gut	pooled	fed	adult			5.00	0.270	
39	gut	pooled	fed	adult			11.00	0.295	
39	gut	pooled	fed	adult			22.00	0.384	
39	gut	pooled	fed	adult		aprotinin	0.00	0.256	1.610
39	gut	pooled	fed	adult		aprotinin	5.00	0.267	
39	gut	pooled	fed	adult		aprotinin	11.00	0.287	
39	gut	pooled	fed	adult		aprotinin	22.00	0.365	
40	sgc	pooled	fed	adult			0.00	0.254	0.861
40	sgc	pooled	fed	adult			5.00	0.368	
40	sgc	pooled	fed	adult			11.00	0.521	
40	sgc	pooled	fed	adult			22.00	0.755	
40	sgc	pooled	fed	adult		aprotinin	0.00	0.256	0.861
40	sgc	pooled	fed	adult		aprotinin	5.00	0.277	
40	sgc	pooled	fed	adult		aprotinin	11.00	0.312	
40	sgc	pooled	fed	adult		aprotinin	22.00	0.399	
40	gut	pooled	fed	adult			0.00	0.253	1.770
40	gut	pooled	fed	adult			5.00	0.280	
40	gut	pooled	fed	adult			11.00	0.297	
40	gut	pooled	fed	adult			22.00	0.391	
40	gut	pooled	fed	adult		aprotinin	0.00	0.259	1.770
40	gut	pooled	fed	adult		aprotinin	5.00	0.265	
40	gut	pooled	fed	adult		aprotinin	11.00	0.283	
40	gut	pooled	fed	adult		aprotinin	22.00	0.356	

A3.2 General Proteinase Assay (Azocasein): Analysis of Variance

A3.2.1 Variate: Total Activity

Source	d.f.	s.s.	m.s.	F-test
Between groups	3	0.00241	0.0008	10.937
Within groups	65	0.00478	0.00007	p=0.0001
Total	68	0.00719		

A3.2.2 Variate: Specific Activity

Source	d.f.	s.s.	m.s.	F-test
Between groups	2	8.0299	4.015	69.396
Within groups	52	3.0085	0.0579	p=0.0001
Total	54	11.0384		

A3.2.3 Variate: Total Activity (effect of DTT and EDTA)

Source	d.f.	s.s.	m.s.	F-test	P value
Tissue	1	0.00006	0.00006	2.614	0.1216
Treatment	2	0.00007	0.00004	1.667	0.2141
Tissue.treatment	2	4.4×10^{-6}	2.2×10^{-6}	0.1001	0.9052
Error	20	0.00045	0.00002		

APPENDIX 4: PROTEASE ASSAY (HEMOGLOBIN), RAW DATA

A4.1 Salivary Gland Extract Data, Absorbance (280 nm)

Rep	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	pH 11
1		0.761	0.762	0.998	0.839	0.749	0.818	0.684	0.588	
2		0.251	0.241	0.061	0.174	0.431	0.598	0.597	0.562	
3		0.318	0.27	0.145	0.287	0.683	0.91	0.905	0.628	
4		0.479	0.285	0.395	0.355	0.403	0.315	0.402	0.343	
5		0.297	0.033	0.289	0.245	0.443	0.3	0.21	0.192	
6		0.337	0.168	0.274	0.308	0.442	0.307	0.201	0.231	
7	0.065	0.192	0.174	0.208	0.242	0.185	0.196	0.216	0.17	0.137
8	0.193	0.312	0.258	0.278	0.242	0.299	0.278	0.254		0.246
9	0.15	0.121	0.496	0.33	0.216	0.258	0.257	0.212	0.189	0.148
10	0.186	0.167	0.636	0.385	0.388	0.38	0.347	0.269	0.234	0.193

A4.2 Midgut Extract Data, Absorbance (280 nm)

Rep	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	pH 11
1		0.912	0.598	0.442	0.487	0.449	0.504	0.436	0.448	
2		0.221	0.681	0.185	0.016	-0.056	-0.059	-0.026	-0.017	
3		0.431	0.961	0.398	0.149	0.147	0.092	0.117	0.043	
5		1.25	0.465	0.394	0.381	0.361	0.281	0.368	0.338	
6		0.592		0.3	0.352	0.472	0.299	0.224	0.215	
7		0.705	0.321			0.474	0.333	0.245	0.234	
8	0.074	0.423	0.292	0.273	0.305	0.32	0.248	0.33	0.234	0.267
9	0.191	0.524	0.444	0.387	0.406	0.409	0.379	0.356	0.378	0.338
10	0.191	0.258	0.77	0.585	0.482	0.477	0.476	0.336	0.276	0.281
11	0.234	0.406	0.895	0.643	0.576	0.506	0.407	0.373	0.271	0.264

A4.3 Secreted Saliva Data, Absorbance (280 nm)

Rep	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	10
1	0.084	0.037	0.032	0.124	0.138	0.182	0.094	0.192
2	0.115	0.172	0.118	0.123	0.192	0.125	0.189	0.089
3	0.075	0.008	0.016	0.019	0.011	0.026	-0.008	0.04
4	0.099	0.079	0.012	0.059	0.05	0.037	0.024	0.075
5	0.121	0.046	0.051	0.203	0.166	0.027	0.028	0.108
6	0.338	0.394	0.25	0.282	0.355	0.353	0.411	0.734
7	0.222	0.225	0.147	0.152	0.154	0.167	0.143	0.388

APPENDIX 5: SPECIFIC SUBSTRATE ASSAYS; RAW DATA AND STATISCAL TESTS

KEY to Terms and Abbreviations:

BApNA: *N*α-benzoyl-DL-arginine *p*-nitroanilide

BTpNA: *N*-benzoyl-L-tyrosine *p*-nitroanalide

SA_2PLNA: *N*-succinyl-ala-ala-pro-leu *p*-nitroanilide

SA_3pNA: *N*-succinyl-ala-ala-ala *p*-nitroanilide

Rep: replicate

Sample: sgc = salivary gland conplex extract

gut = midgut extract

con = midgut luminal contents

sal = secreted saliva

blank = buffer only

Time: time (hr) of absorbance reading after addition of substrate

Absorbance: Absorbance of sample at 405 nm

Prot. conc.: Protein concentration of the sample (mg/ml)

Activity: Protease activity of the sample, measured as change in absorbance per min per mg protein x 10^{-3}

pNA: nmol of *p*-nitroanilide released per min per mg protein

A5.1 BApNA

A5.1.1 BApNA Assay, Raw Data

Rep	Sample	Time	Absorbance
1	sgc	0.0	0.16
1	sgc	1	0.193
1	sgc	2	0.21
1	sgc	4	0.223
1	sgc	7.5	0.256
1	sgc	10.5	0.276
1	gut	0.05	0.17
1	gut	1	0.337
1	gut	2	0.392
1	gut	4	0.498
1	gut	7.5	0.706
1	gut	10.5	0.811
1	con	0.05	0.226
1	con	1	0.28
1	con	2	0.388
1	con	4	0.68
1	con	7.5	1.207
1	con	10.5	1.513
1	sal	0.05	0.11
1	sal	1	0.098
1	sal	2	0.096
1	sal	4	0.085
1	sal	7.5	0.082
1	sal	10.5	0.083
2	sgc	0.05	0.088
2	sgc	1	0.083
2	sgc	2	0.099
2	sgc	4	0.104
2	sgc	7.5	0.134
2	sgc	10.5	0.158
2	gut	0.05	0.128
2	gut	1	0.241
2	gut	2	0.267
2	gut	4	0.286
2	gut	7.5	0.355
2	gut	10.5	0.387
2	con	0.05	0.121
2	con	1	0.218
2	con	2	0.36
2	con	4	0.656
2	con	7.5	1.259
2	con	10.5	1.618
3	sgc	0.05	0.104
3	sgc	1	0.111
3	sgc	2	0.128
3	sgc	4	0.139
3	sgc	7.5	0.17
3	sgc	10.5	0.193
3	gut	0.05	0.233
3	gut	1	0.377

Rep	Sample	Time	Absorbance
3	gut	2	0.45
3	gut	4	0.566
3	gut	7.5	0.798
3	gut	10.5	0.919
3	con	0.05	0.179
3	con	1	0.317
3	con	2	0.499
3	con	4	0.907
3	con	7.5	1.755
3	con	10.5	2.238
4	sgc	0.05	0.085
4	sgc	1	0.117
4	sgc	2	0.144
4	sgc	4	0.192
4	sgc	7.5	0.34
4	sgc	10.5	0.523
4	gut	0.05	0.13
4	gut	1	0.284
4	gut	2	0.312
4	gut	4	0.34
4	gut	7.5	0.425
4	gut	10.5	0.462
4	con	0.05	0.166
4	con	1	0.201
4	con	2	0.274
4	con	4	0.43
4	con	7.5	0.758
4	con	10.5	0.953
5	sgc	0.05	0.123
5	sgc	1	0.156
5	sgc	2	0.173
5	sgc	4	0.188
5	sgc	7.5	0.236
5	sgc	10.5	0.266
5	gut	0.05	0.258
5	gut	1	0.474
5	gut	2	0.617
5	gut	4	0.856
5	gut	7.5	1.276
5	gut	10.5	1.53
5	con	0.05	0.425
5	con	1	0.839
5	con	2	1.365
5	con	4	2.292
5	con	7.5	2.999
5	con	10.5	3.075
6	sgc	0.05	0.208
6	sgc	1	0.199
6	sgc	2	0.203
6	sgc	4	0.215
6	sgc	7.5	0.265
6	sgc	10.5	0.333

Rep	Sample	Time	Absorbance
6	gut	0.05	0.351
6	gut	1	0.376
6	gut	2	0.408
6	gut	4	0.454
6	gut	7.5	0.511
6	gut	10.5	0.551
6	con	0.05	0.385
6	con	1	0.381
6	con	2	0.405
6	con	4	0.43
6	con	7.5	0.537
6	con	10.5	0.617

A5.1.2 BApNA Assay, Calculations

Rep	Sample	Prot. conc.	Activity	pNA
1	sgc	0.951	3.571	0.107
1	gut	2.56	7.500	0.224
1	con	0.424	102.359	3.058
1	sal	0.001	-0.767	0
2	sgc	1.05	2.286	0.0683
2	gut	2.66	2.556	0.0764
2	con	0.36	137.778	4.117
3	sgc	1.09	2.569	0.0768
3	gut	2.74	7.660	0.229
3	con	0.705	96.884	2.895
4	sgc	1.24	10.968	0.328
4	gut	2.24	3.839	0.115
4	con	0.363	72.528	2.257
5	sgc	1.48	2.838	0.0848
5	gut	3.46	11.445	0.342
5	con	3.78	23.016	0.688
6	sgc	3.16	1.266	0.0378
6	gut	3.88	1.649	0.0493
6	con	0.985	7.911	0.268

A5.2 BTpNA

A5.2.1 BTpNA Assay, Raw Data

Rep	Sample	Time	Absorbance
1	sgc	0	0.043
1	sgc	20	0.414
1	gut	0	0.063
1	gut	20	0.059
1	blank	0	0.035
1	blank	20	0.027
2	sgc	0	0.047
2	sgc	20	0.404
2	gut	0	0.066
2	gut	20	0.074
3	sgc	0	0.041
3	sgc	20	0.221
3	gut	0	0.063
3	gut	20	0.056
4	sgc	0	0.047
4	sgc	20	0.332
4	gut	0	0.053
4	gut	20	0.065
5	sgc	0	0.044
5	sgc	20	0.462
5	gut	0	0.061
5	gut	20	0.065
6	sgc	0	0.045
6	sgc	20	0.239
6	gut	0	0.078
6	gut	20	0.070

A5.2.2 BT pNA Assay, Calculations

Rep	Sample	Prot. conc.	Activity	pNA
1	sgc	1.874	1.650	0.493
1	gut	3.036	-0.011	-0.003
2	sgc	2.160	1.377	0.412
2	gut	3.612	0.018	0.006
3	sgc	1.416	1.059	0.317
3	gut	3.104	-0.019	-0.006
4	sgc	2.480	0.958	0.286
4	gut	3.172	0.032	0.009
5	sgc	1.756	1.984	0.593
5	gut	3.276	0.010	0.003
6	sgc	2.240	0.722	0.216
6	gut	3.476	-0.019	-0.006

A5.3 SA_2PLNA

A5.3.1 SA_2PLNA Assay, Raw Data

Replicate	Sample	Time	Absorbance
1	sgc	0.083	0.074
1	sgc	0.5	0.303
1	sgc	1	0.629
1	sgc	2	1.365
1	sgc	3.5	2.27
1	sgc	4	2.438
1	gut	0.083	0.086
1	gut	0.5	0.105
1	gut	1	0.128
1	gut	2	0.152
1	gut	3.5	0.169
1	gut	4	0.171
1	con	0.083	0.289
1	con	0.5	0.275
1	con	1	0.259
1	con	2	0.282
1	con	3.5	0.277
1	con	4	0.275
1	sal	0.083	0.054
1	sal	0.5	0.065
1	sal	1	0.084
1	sal	2	0.091
1	sal	3.5	0.1
1	sal	4	0.102
2	sgc	0.083	0.097
2	sgc	0.5	0.245
2	sgc	1	0.505
2	sgc	2	1.236
2	sgc	3.5	2.176
2	sgc	4	2.35
2	gut	0.083	0.152
2	gut	0.5	0.168
2	gut	1	0.208
2	gut	2	0.351
2	gut	3.5	0.597
2	gut	4	0.659
2	con	0.083	0.162
2	con	0.5	0.16
2	con	1	0.167
2	con	2	0.182
2	con	3.5	0.213
2	con	4	0.226
3	sgc	0.083	0.075
3	sgc	0.5	0.203
3	sgc	1	0.445
3	sgc	2	1.259
3	sgc	3.5	2.494
3	sgc	4	2.657
3	gut	0.083	0.094
3	gut	0.5	0.111

Replicate	Sample	Time	Absorbance
3	gut	1	0.168
3	gut	2	0.342
3	gut	3.5	0.514
3	gut	4	0.559
3	con	0.083	0.193
3	con	0.5	0.219
3	con	1	0.266
3	con	2	0.353
3	con	3.5	0.474
3	con	4	0.504
4	sgc	0.083	0.091
4	sgc	0.5	0.308
4	sgc	1	0.697
4	sgc	2	1.658
4	sgc	3.5	2.535
4	sgc	4	2.649
4	gut	0.083	0.135
4	gut	0.5	0.142
4	gut	1	0.18
4	gut	2	0.237
4	gut	3.5	0.273
4	gut	4	0.276
4	con	0.083	0.132
4	con	0.5	0.131
4	con	1	0.118
4	con	2	0.128
4	con	3.5	0.137
4	con	4	0.14
5	sgc	0.083	0.064
5	sgc	0.5	0.124
5	sgc	1	0.235
5	sgc	2	0.594
5	sgc	3.5	1.309
5	sgc	4	1.511
5	gut	0.083	0.113
5	gut	0.5	0.114
5	gut	1	0.132
5	gut	2	0.188
5	gut	3.5	0.222
5	gut	4	0.227
5	con	0.083	0.194
5	con	0.5	0.186
5	con	1	0.191
5	con	2	0.204
5	con	3.5	0.216
5	con	4	0.219
6	sgc	0.083	0.115
6	sgc	0.5	0.162
6	sgc	1	0.25
6	sgc	2	0.52
6	sgc	3.5	1.08
6	sgc	4	1.25

Replicate	Sample	Time	Absorbance
6	gut	0.083	0.202
6	gut	0.5	0.211
6	gut	1	0.299
6	gut	2	0.485
6	gut	3.5	0.743
6	gut	4	0.814
6	con	0.083	0.148
6	con	0.5	0.136
6	con	1	0.144
6	con	2	0.156
6	con	3.5	0.166
6	con	4	0.173

A5.3.2 SA_2PLNA Assay, Calculations

Rep	Sample	Prot. conc.	Activity	pNA
1	sgc	0.875	237.670	7.101
1	gut	1.238	5.493	0.164
1	con	1.63	0	0
1	sal	0.00648	0.297	8.861
2	sgc	1.28	158.125	4.725
2	gut	2.8	16.357	0.489
2	con	0.555	10.432	0.312
3	sgc	0.944	250.636	7.489
3	gut	2.1	20.095	0.600
3	con	1.3	20.923	0.625
4	sgc	1.17	196.239	5.863
4	gut	2.68	4.776	0.143
4	con	0.169	5.917	0.177
5	sgc	1.02	126.078	3.767
5	gut	2.86	3.776	0.113
5	con	0.383	6.771	0.202
6	sgc	1.3	77.231	2.308
6	gut	3.32	16.627	0.496
6	con	0.625	4.153	0.124

A5.4 SA_3pNA

A5.4.1 SA_3pNA Assay, Raw Data

Replicate	Sample	Time	Absorbance
1	sgc	0.083	0.109
1	sgc	1.167	0.112
1	sgc	3	0.136
1	sgc	4	0.155
1	sgc	11.12	0.41
1	sgc	23.5	0.869
1	gut	0.083	0.149
1	gut	1.167	0.16
1	gut	3	0.187
1	gut	4	0.195
1	gut	11.12	0.231
1	gut	23.5	0.266
1	con	0.083	0.209
1	con	1.167	0.206
1	con	3	0.203
1	con	4	0.199
1	con	11.12	0.212
1	con	23.5	0.279
1	sal	0.083	0.073
1	sal	1.167	0.118
1	sal	3	0.125
1	sal	4	0.121
1	sal	11.12	0.135
1	sal	23.5	0.126
2	sgc	0.083	0.092
2	sgc	1.167	0.097
2	sgc	3	0.116
2	sgc	4	0.141
2	sgc	11.12	0.426
2	sgc	23.5	0.863
2	gut	0.083	0.134
2	gut	1.167	0.168
2	gut	3	0.2
2	gut	4	0.217
2	gut	11.12	0.276
2	gut	23.5	0.349
2	con	0.083	0.142
2	con	1.167	0.139
2	con	3	0.136
2	con	4	0.133
2	con	11.12	0.138
2	con	23.5	0.162
3	sgc	0.083	0.091
3	sgc	1.167	0.099
3	sgc	3	0.097
3	sgc	4	0.098
3	sgc	11.12	0.199
3	sgc	23.5	0.519

Replicate	Sample	Time	Absorbance
3	gut	0.083	0.122
3	gut	1.167	0.15
3	gut	3	0.161
3	gut	4	0.165
3	gut	11.12	0.183
3	gut	23.5	0.185
3	con	0.083	0.205
3	con	1.167	0.199
3	con	3	0.2
3	con	4	0.2
3	con	11.12	0.218
3	con	23.5	0.288
4	sgc	0.083	0.096
4	sgc	1.167	0.095
4	sgc	3	0.097
4	sgc	4	0.098
4	sgc	11.12	0.133
4	sgc	23.5	0.257
4	gut	0.083	0.211
4	gut	1.167	0.21
4	gut	3	0.224
4	gut	4	0.227
4	gut	11.12	0.252
4	gut	23.5	0.267
4	con	0.083	0.138
4	con	1.167	0.158
4	con	3	0.162
4	con	4	0.159
4	con	11.12	0.18
4	con	23.5	0.225
5	sgc	0.083	0.07
5	sgc	1.167	0.071
5	sgc	3	0.074
5	sgc	4	0.082
5	sgc	11.12	0.165
5	sgc	23.5	0.366
5	gut	0.083	0.194
5	gut	1.167	0.204
5	gut	3	0.219
5	gut	4	0.225
5	gut	11.12	0.276
5	gut	23.5	0.36
5	con	0.083	0.339
5	con	1.167	0.314
5	con	3	0.313
5	con	4	0.317
5	con	11.12	0.368
5	con	23.5	0.541
6	sgc	0.083	0.118
6	sgc	1.167	0.113
6	sgc	3	0.111
6	sgc	4	0.118

Replicate	Sample	Time	Absorbance
6	sgc	11.12	0.136
6	sgc	23.5	0.201
6	gut	0.083	0.172
6	gut	1.167	0.183
6	gut	3	0.197
6	gut	4	0.199
6	gut	11.12	0.218
6	gut	23.5	0.228
6	con	0.083	0.308
6	con	1.167	0.296
6	con	3	0.284
6	con	4	0.281
6	con	11.12	0.282
6	con	23.5	0.388

A5.4.2 SA_3pNA Assay, Calculations

Rep	Sample	Prot.conc.	Activity	pNA
1	sgc	1.76	6.364	0.190
1	gut	3.58	0.447	0.0134
1	con	0.82	1.220	0.0364
1	sal	0.00136	0.571	17.060
2	sgc	2.09	5.429	0.162
2	gut	4.08	0.686	0.0205
2	con	0.179	2.235	0.0668
3	sgc	2.31	2.672	0.180
3	gut	3.24	0.185	0.00553
3	con	0.88	1.364	0.0408
4	sgc	1.04	2.308	0.0690
4	gut	2.94	0.272	0.00813
4	con	0.42	2.381	0.0711
5	sgc	1.59	2.767	0.0827
5	gut	3.4	0.706	0.0211
5	con	1.14	2.807	0.0839
6	sgc	1.18	1.017	0.0304
6	gut	3.34	0.240	0.0716
6	con	1.175	1.020	0.0305

A5.5 Analysis of Variance (ANOVA)

Variate: nmol pNA min^{-1} mg^{-1}

Source	d.f.	s.s.	m.s.	F-test	P value
tissue	1	21.021	21.021	36.497	0.0001
substrate	2	31.812	15.906	27.615	0.0001
tissue.substrate	5	93.117	18.623	32.334	0.0001
Error	55	31.679	0.576		

APPENDIX 6
ANALYSIS OF PCR AMPLICON C63.27

Figure A6.1: Nucleotide and predicted amino acid sequences of c63.27

```
1    AATTCACTAAGTGATTCCACGAGTTTTTGACACAGCCACATGTTTGTGCT
1    N  S  L  S  D  S  T  S  F  *  H  S  H  M  F  V  L

51   GGAGCAAGAAGAGTACACCCGCGAAGGTATTGAATGGGCGTTCATTGATT
18    E  Q  E  E  Y  T  R  E  G  I  E  W  A  F  I  D  F

101  TTGGAATGGATTTATCCGCCTGCATTGAGCTCATTGAAAAGCCCATGGGT
35     G  M  D  L  S  A  C  I  E  L  I  E  K  P  M  G

151  ATCTTGTCCATCCTTGAGGAAGAATCTATGTTCCCCAAGGCTACTGACAA
51   I  L  S  I  L  E  E  E  S  M  F  P  K  A  T  D  K

201  GACCTTTGAGGACAAACTGATCACCAACCACTTGGGCAAATCTCCCAAC
68    T  F  E  D  K  L  I  T  N  H  L  G  K  S  P  N
```

Table A6.1: Most similar sequences to green mirid PCR amplicon c63.27, determined by FastA searching of non-redundant nucleotide databases.

Homologous Sequence	% nucleotide (nt) identity
Quail slow myosin heavy chain 3	77.3% over 255 nt
Chicken embryonic myosin heavy chain gene	76.7% over 249 nt
Homo sapiens beta-myosin heavy chain	75.7% over 255 nt

Table A6.1: Most similar sequences to the predicted amino acid sequence of mirid PCR amplicon c63.27, determined by FastA searching of non-redundant protein databases.

Homologous Sequence	% amino acid (aa) identity
chicken myosin S-1 heavy chain, cardiac muscle	79.0% over 76 aa
D.melanogaster myosin heavy chain gene	81.1% over 74 aa
Human cardiac beta myosin heavy chain	77.6% over 76 aa